TITLE

PROCESS FOR THE BIOLOGICAL PRODUCTION OF 1,3-PROPANEDIOL WITH HIGH YIELD

This application claims the benefit of U.S. Provisional Application No. 60/416,192, filed October 4, 2003.

FIELD OF INVENTION

This invention comprises a process for the bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism.

BACKGROUND

1,3-Propanediol is a monomer having potential utility in the production of polyester fibers and the manufacture of polyurethanes and cyclic compounds.

A variety of chemical routes to 1,3-propanediol are known. For example ethylene oxide may be converted to 1,3-propanediol over a catalyst in the presence of phosphine, water, carbon monoxide, hydrogen and an acid, by the catalytic solution phase hydration of acrolein followed by reduction, or from compounds such as glycerol, reacted in the presence of carbon monoxide and hydrogen over catalysts having atoms from group VIII of the periodic table. Although it is possible to generate 1,3-propanediol by these methods, they are expensive and generate waste streams containing environmental pollutants.

It has been known for over a century that 1,3-propanediol can be produced from the fermentation of glycerol. Bacterial strains able to produce 1,3-propanediol have been found, for example, in the groups *Citrobacter*, *Clostridium*, *Enterobacter*, *Ilyobacter*, *Klebsiella*, *Lactobacillus*, and *Pelobacter*. In each case studied, glycerol is converted to 1,3-propanediol in a two step, enzyme catalyzed reaction sequence. In the first step, a dehydratase catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA) and water, Equation 1. In the second step, 3-HPA is reduced to 1,3-propanediol by a NAD^+-linked oxidoreductase, Equation 2. The 1,3-propanediol is not metabolized further and, as a result,

$$\text{Glycerol} \rightarrow \text{3-HPA} + H_2O \qquad \text{(Equation 1)}$$

$$\text{3-HPA} + \text{NADH} + H^+ \rightarrow \text{1,3-Propanediol} + NAD^+ \qquad \text{(Equation 2)}$$

accumulates in the media. The overall reaction consumes a reducing equivalent in the form of a cofactor, reduced β-nicotinamide adenine dinucleotide (NADH), which is oxidized to nicotinamide adenine dinucleotide (NAD^+).

In *Klebsiella pneumonia*, *Citrobacter freundii*, and *Clostridium pasteurianum*, the genes encoding the three structural subunits of glycerol dehydratase (*dhaB1-3* or *dhaB, C and E*) are located adjacent to a gene encoding a specific 1,3-propanediol oxidoreductase (*dhaT*). Although the genetic organization differs somewhat among these microorganisms, these genes are clustered in a group which also comprises *orfX* and *orfZ* (genes encoding a dehydratase reactivation factor for glycerol dehydratase), as well as *orfY* and *orfW* (genes of unknown function). The specific 1,3-propanediol oxidoreductases (*dhaT*'s) of these microorganisms are known to belong to the family of type III alcohol dehydrogenases; each exhibits a conserved iron-binding motif and has a preference for the NAD^+/NADH linked interconversion of 1,3-propanediol and 3-HPA. However, the NAD^+/NADH linked interconversion of 1,3-propanediol and 3-HPA is also catalyzed by alcohol dehydrogenases which are not specifically linked to dehydratase enzymes (for example, horse liver and baker's yeast alcohol dehydrogenases (E.C. 1.1.1.1)), albeit with less efficient kinetic parameters. Glycerol dehydratase (E.C. 4.2.1.30) and diol [1,2-propanediol] dehydratase (E.C. 4.2.1.28) are related but distinct enzymes that are encoded by distinct genes. Diol dehydratase genes from *Klebsiella oxytoca* and *Salmonella typhimurium* are similar to glycerol dehydratase genes and are clustered in a group which comprises genes analogous to *orfX* and *orfZ* (Daniel *et al.*, *FEMS Microbiol. Rev. 22*, 553 (1999); Toraya and Mori, *J. Biol. Chem. 274*, 3372 (1999); GenBank AF026270).

The production of 1,3-propanediol from glycerol is generally performed under anaerobic conditions using glycerol as the sole carbon source and in the absence of other exogenous reducing equivalent acceptors. Under these conditions, in e.g., strains of *Citrobacter*, *Clostridium*, and *Klebsiella*, a parallel pathway for glycerol operates which first involves oxidation of glycerol to dihydroxyacetone (DHA) by a NAD^+- (or $NADP^+$-) linked glycerol dehydrogenase, Equation 3. The DHA, following phosphorylation to dihydroxyacetone phosphate (DHAP) by a DHA kinase (Equation 4),

$$\text{Glycerol} + NAD^+ \rightarrow \text{DHA} + NADH + H^+ \qquad \text{(Equation 3)}$$

$$\text{DHA} + ATP \rightarrow \text{DHAP} + ADP \qquad \text{(Equation 4)}$$

becomes available for biosynthesis and for supporting ATP generation via e.g., glycolysis. In contrast to the 1,3-propanediol pathway, this pathway may provide carbon and energy to the cell and produces rather than consumes NADH.

In *Klebsiella pneumoniae* and *Citrobacter freundii*, the genes encoding the functionally linked activities of glycerol dehydratase (*dhaB*), 1,3-propanediol oxidoreductase (*dhaT*), glycerol dehydrogenase (*dhaD*), and dihydroxyacetone kinase (*dhaK*) are encompassed by the *dha* regulon. The dha regulon, in *Klebsiella pneumoniae* and *Citrobacter freundii*, also encompasses a gene encoding a transcriptional activator protein (*dhaR*). The *dha* regulons from *Citrobacter* and *Klebsiella* have been expressed in *Escherichia coli* and have been shown to convert glycerol to 1,3-propanediol.

Neither the chemical nor biological methods described above for the production of 1,3-propanediol are well suited for industrial scale production since the chemical processes are energy intensive and the biological processes are limited to relatively low titer from the expensive starting material, glycerol. These drawbacks could be overcome with a method requiring low energy input and an inexpensive starting material such as carbohydrates or sugars, or by increasing the metabolic efficiency of a glycerol process. Development of either method will require the ability to manipulate the genetic machinery responsible for the conversion of sugars to glycerol and glycerol to 1,3-propanediol.

Biological processes for the preparation of glycerol are known. The overwhelming majority of glycerol producers are yeasts but some bacteria, other fungi, and algae are also known. Both bacteria and yeasts produce glycerol by converting glucose or other carbohydrates through the fructose-1,6-bisphosphate pathway in glycolysis or the Embden Meyerhof Parnas pathway. Dihydroxyacetone phosphate is converted to glycerol-3-phosphate by the action of glycerol-3-phosphate dehydrogenase, and glycerol-3-phosphate is converted to glycerol by the action of glycerol-3-phosphatase.

The gene encoding glycerol-3-phosphate dehydrogenase (DAR1, GPD1) has been cloned and sequenced from *S. diastaticus* (Wang *et al.*, *J. Bact. 176*, 7091-7095 (1994)). The DAR1 gene was cloned into a shuttle vector and used to transform *E. coli* where expression produced active enzyme. Wang *et al.* (*supra*) recognize that DAR1 is regulated by the cellular osmotic environment but do not suggest how the gene might be used to enhance 1,3-propanediol production in a recombinant microorganism.

Other glycerol-3-phosphate dehydrogenase enzymes have been isolated: for example, sn-glycerol-3-phosphate dehydrogenase has been cloned and sequenced from *Saccharomyces cerevisiae* (Larason *et al.*, *Mol. Microbiol. 10*, 1101 (1993)) and Albertyn *et al.* (*Mol. Cell. Biol. 14*, 4135 (1994)) teach the cloning of GPD1 encoding a glycerol-3-phosphate dehydrogenase from *Saccharomyces cerevisiae*. Like Wang *et al.* (*supra*), both Albertyn *et al.* and Larason *et al.* recognize the osmosensitivity of the regulation of this gene but do not suggest how the gene might be used in the production of 1,3-propanediol in a recombinant microorganism.

As with G3PDH, glycerol-3-phosphatase has been isolated from *Saccharomyces cerevisiae* and the protein identified as being encoded by the GPP1 and GPP2 genes (Norbeck *et al.*, *J. Biol. Chem. 271*, 13875 (1996)). Like the genes encoding G3PDH, it appears that GPP2 is osmosensitive.

WO 9634961 and Hernandez-Montalvo *et al.* (*Appl. Microbiol. Biotechnol. 57*:186-191 (2001) describe *E. coli* strains that have "PTS" minus/glucose plus phenotypes. EP 1170376 A1 discloses deletion of a gene for NADH dehydratase II to improve energy efficiency. WO 2001016346 describes the utility of "aldehyde dehydrogenase A" and "aldehyde dehydrogenase B" for the production of 3-hydroxypropionic acid.

WO 9635796 (US 5,686,276, E. I. du Pont de Nemours and Company ("DuPont")) discloses a method for the production of 1,3-propanediol from a carbon substrate other than glycerol or dihydroxyacetone (especially, e.g., glucose), using a single microorganism comprising a dehydratase activity. WO 9928480 (DuPont) discloses a similar method with advantages derived from expressing exogenous activities of one or both of glycerol-3-phosphate dehydrogenase and

glycerol-3-phosphate phosphatase while disrupting one or both of endogenous activities glycerol kinase and glycerol dehydrogenase. WO 9821339 (US 6,013,494, DuPont) describes a process for the production of 1,3-propanediol using a single microorganism comprising exogenous glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate phosphatase, dehydratase, and 1,3-propanediol oxidoreductase (e.g., *dhaT*). WO 9821341 (US 6,136,576, DuPont) discloses a method for the production of 1,3-propanediol comprising a recombinant microorganism further comprising a dehydratase and protein X (later identified as being a dehydratase reactivation factor peptide). WO 2001012833 (DuPont) describes an improvement to the process where a significant increase in titer (grams product per liter) is obtained by virtue of a non-specific catalytic activity (distinguished from 1,3-propanediol oxidoreductase encoded by *dhaT*) to convert 3-hydroxypropionaldehyde to 1,3-propanediol. US 10/420587 (2003) (US 60/374931 (2002)DuPont)) discloses vectors and plasmids useful for the production of 1,3-propanediol. The DuPont applications are incorporated by reference in the instant specification as though set forth in their entirety herein.

The biological production of 1,3-propanediol requires glycerol as an intermediate substrate for a two-step sequential reaction in which a dehydratase enzyme (typically a coenzyme B_{12}-dependent dehydratase) converts glycerol to 3-hydroxypropionaldehyde, which is then reduced to 1,3-propanediol by a NADH- (or NADPH-) dependent oxidoreductase. The complexity of the cofactor requirements necessitates the use of a whole cell catalyst for an industrial process that utilizes this reaction sequence for the production of 1,3-propanediol.

A specific deficiency in the biological processes leading to the production of 1,3-propanediol from glucose has been the low yield of the product achieved via fermentation. WO 2001012833 (DuPont) describes weight yields of 1,3-propanediol from glucose within the range of 24% and 35%. The problem that remains to be solved is how to biologically produce 1,3-propanediol, with high yield and by a single microorganism, from an inexpensive carbon substrate such as glucose or other sugars.

SUMMARY OF THE INVENTION

Applicants have solved the stated problem. The present invention provides for bioconverting a fermentable carbon source to 1,3-propanediol at higher yield than previously obtained and with the use of a single

microorganism. The yield obtained is greater than 35%, and preferably greater than 40%. Glucose is used as a model substrate and *Escherichia coli* is used as the model host microorganism with the useful genetic modifications and disruptions detailed herein.

Applicants have provided an *E. coli* strain comprising:

a) a disrupted endogenous phosphoenolpyruvate-glucose phosphotransferase system preventing expression of active PEP-glucose phosphotransferase system proteins;

b) an up regulated endogenous *galP* gene encoding active galactose-proton symporter;

c) an up regulated endogenous *glk* gene encoding active glucokinase; and

d) a down regulated endogenous *gapA* gene encoding active glycerolaldehyde 3-phosphate dehydrogenase.

Applicants have also provided an *E. coli* strain described above wherein the disrupted endogenous phosphoenolpyruvate-glucose phosphotransferase system comprises one or more of

a1) a disrupted endogenous *ptsH* gene preventing expression of active phosphocarrier protein;

a2) a disrupted endogenous *ptsI* gene preventing expression of active phosphoenolpyruvate-protein phosphotransferase; and

a3) a disrupted endogenous *crr* gene preventing expression of active glucose-specific IIA component.

The *E. coli* embodiments described above can further comprise one or more of

e) a disrupted endogenous *arcA* gene preventing expression of active aerobic respiration control protein;

f) an up regulated endogenous *ppc* gene encoding active phosphoenolpyruvate carboxylase;

g) an up regulated endogenous *btuR* gene encoding active cob(I)alamin adenosyltransferase; and

h) an up regulated *yqhD* gene encoding active alcohol dehydrogenase.

The *E. coli* embodiments described above can further comprise one or more of

i) a disrupted endogenous *mgsA* gene preventing the expression of active methylglyoxal synthase;

j) a disrupted endogenous *ackA* gene preventing the expression of active acetate kinase;

k) a disrupted endogenous *pta* gene preventing the expression of active phosphotrasacetylase;

l) a disrupted endogenous *aldA* gene preventing the expression of active aldehyde dehydrogenase A; and

m) a disrupted endogenous *aldB* gene preventing the expression of active aldehyde dehydrogenase B.

The *E. coli* embodiments described above can further comprise one or more of:

n) a disrupted endogenous *edd* gene preventing expression of active phosphogluconate dehydratase;

o) a disrupted endogenous *glpK* gene preventing expression of active glycerol kinase; and

p) a disrupted endogenous *gldA* gene preventing expression of active NADH-dependent glycerol dehydrogenase.

Additionally, 1,3-propanediol can be bioproduced by contacting an *E. coli* strain described herein with a suitable carbon substrate such as glucose under suitable conditions for fermentation. In addition, 1,3-propanediol can be bioproduced by contacting an *E. coli* strain described herein, the *E. coli* strain further comprising an active:

(i) glycerol-3-phosphate dehydrogenase;

(ii) glycerol-3-phosphatase;

(iii) dehydratase; and

(iv) dehydratase reactivating activity;

with a suitable carbon substrate under suitable conditions.

Additionally, any of the embodiments disclosed above may also include the constructs pSYCO101, pSYCO103, pSYCO106, pSYCO109 or their corresponding nucleotide sequences SEQ ID NOs:65, 66, 67, or 68.

BRIEF DESCRIPTION OF THE DRAWINGS, SEQUENCE DESCRIPTIONS, AND BIOLOGICAL DEPOSITS

The invention can be more fully understood from the following detailed description, the Figure 1, the accompanying sequence listing and descriptions, and biological deposits that form parts of this application.

Figure 1 shows 1,3-propanediol production compared as between two fermentations run essentially as described in GENERAL METHODS. In one case, the strain used was FMP'::Km/pSYCO103. In the other case, the strain used was FMP/pSYCO103.

The 68 sequence descriptions and the sequence listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures – the Sequence Rules") and will be consistent with World Intellectual Property Organization (WIPO) Standard ST2.5 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administration Instructions). The Sequence Descriptions contain the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IYUB standards described in *Nucleic Acids Res. 13*, 3021-3030 (1985) and in the *Biochemical Journal 219*, 345-373 (1984) which are herein incorporated by reference.

SEQ ID NO:1 is the partial nucleotide sequence of pLoxCat27 encoding the *loxP*511-Cat-*loxP*511 cassette.

SEQ ID NO:2-3 are oligonucleotide primers used to construct the *arcA* disruption.

SEQ ID NOs:4-5 are oligonucleotide primers used to confirm disruption of *arcA*.

SEQ ID NO:6 is the partial nucleotide sequence of pLoxCat1 encoding the *loxP*-Cat-*loxP* cassette.

SEQ ID NOs:7-8 are oligonucleotide primers used to construct pR6KgalP, the template plasmid for trc promoter replacement of the chromosomal *galP* promoter.

SEQ ID NOs:9-10 are oligonucleotide primers used to construct pR6Kglk, the template plasmid for trc promoter replacement of the chromosomal *glk* promoter.

SEQ ID NO:11 is the nucleotide sequence of the *loxP*-Cat-*loxP*–Trc cassette.

SEQ ID NOs:12-13 are oligonucleotide primers used to confirm integration of SEQ ID NO:11 for replacement of the chromosomal *galP* promoter.

SEQ ID NOs:14-15 are oligonucleotide primers used to confirm integration of SEQ ID NO:11 for replacement of the chromosomal *glk* promoter.

SEQ ID NOs:16-17 are oligonucleotide primers used to construct the *edd* disruption.

SEQ ID NOs:18-19 are oligonucleotide primers used to confirm disruption of *edd*.

SEQ ID NOs:20 is the nucleotide sequence for the selected trc promoter controlling glk expression.

SEQ ID NOs:21 is the partial nucleotide sequence for the standard trc promoter.

SEQ ID NOs:22-23 are the oligonucleotide primers used for amplification of *gapA*.

SEQ ID NOs:24-25 are the oligonucleotide primers used to alter the start codon of *gapA* to GTG.

SEQ ID NOs:26-27 are the oligonucleotide primers used to alter the start codon of *gapA* to TTG.

SEQ ID NO:28 is the nucleotide sequence for the short 1.5 GI promoter.

SEQ ID NOs:29-30 are oligonucleotide primers used for replacement of the chromosomal *gapA* promoter with the short 1.5 GI promoter.

SEQ ID NO:31 is the nucleotide sequence for the short 1.20 GI promoter.

SEQ ID NO:32 is the nucleotide sequence for the short 1.6 GI promoter.

SEQ ID NOs:33-34 are oligonucleotide primers used for replacement of the chromosomal *gapA* promoter with the short 1.20 GI promoter.

SEQ ID NO:35 is the oligonucleotide primer with SEQ ID NO 33 that is used for replacement of the chromosomal *gapA* promoter with the short 1.6 GI promoter.

SEQ ID NOs:36-37 are oligonucleotide primers used to construct the *mgsA* disruption.

SEQ ID NOs:38-39 are oligonucleotide primers used to confirm disruption of *mgsA*.

SEQ ID NOs:40-41 are oligonucleotide primers used for replacement of the chromosomal *ppc* promoter with the short 1.6 GI promoter.

SEQ ID NO:42 is an oligonucleotide primer used to confirm replacement of the *ppc* promoter.

SEQ ID NOs:43-44 are oligonucleotide primers used for replacement of the chromosomal *yciK-btuR* promoter with the short 1.6 GI promoter.

SEQ ID NOs:45-46 are oligonucleotide primers used to confirm replacement of the *yciK-btuR* promoter.

SEQ ID NOs:47-48 are oligonucleotide primers used for replacement of the chromosomal *yqhD* promoter with the short 1.6 GI promoter.

SEQ ID NO:49 is an oligonucleotide primer used to confirm replacement of the *yqhD* promoter.

SEQ ID NOs:50-51 are oligonucleotide primers used to construct the *pta-ackA* disruption.

SEQ ID NOs:52-53 are oligonucleotide primers used to confirm disruption of *pta-ackA*.

SEQ ID NOs:54-55 are oligonucleotide primers used to construct the *ptsHIcrr* disruption.

SEQ ID NO:56 is an oligonucleotide primer used to confirm disruption of *ptsHIcrr*.

SEQ ID NOs:57-58 are oligonucleotide primers used to construct the *aldA* disruption.

SEQ ID NOs:59-60 are oligonucleotide primers used to confirm disruption of *aldA*.

SEQ ID NOs:61-62 are oligonucleotide primers used to construct the *aldB* disruption.

SEQ ID NOs:63-64 are oligonucleotide primers used to confirm disruption of *aldB*.

SEQ ID NO:65 is the nucleotide sequence for the pSYCO101 plasmid.

SEQ ID NO:66 is the nucleotide sequence for the pSYCO103 plasmid.

SEQ ID NO:67 is the nucleotide sequence for the pSYCO106 plasmid.

SEQ ID NO:68 is the nucleotide sequence for the pSYCO109 plasmid.

Applicants have made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure.

Depositor Identification Reference	Int'l Depository Designation	Date of Deposit
Transformed *E. coli* DH5α containing a portion of the Klebsiella genome encoding the glycerol dehydratase enzyme	ATCC 69789	18 April 1995
Transformed *E. coli* DH5α containing cosmid pKP4 containing a portion of Klebsiella genome encoding a diol dehydratase enzyme	ATCC 69790	18 April 1995
E. coli MSP33.6	ATCC 98598	25 November 1997
glpK mutant *E. coli* RJF10m	ATCC 98597	25 November 1997
Escherichia coli: RJ8n	ATCC PTA4216	9 April 2002
Escherichia coli: FMP'::Km	ATCC PTA-4732	28 September 2002

The deposit(s) will be maintained in the indicated international depository for at least 30 years and will be made available to the public upon the grant of a patent disclosing it. The availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by government action.

As used herein, "ATCC" refers to the American Type Culture Collection international depository located 10801 University Blvd., Manassas, VA 20110-2209 U.S.A. The "ATCC No." is the accession number to cultures on deposit with the ATCC.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for an improved process for bioconverting a fermentable carbon source directly to 1,3-propanediol using a single microorganism. The method is characterized by improved 1,3-propanediol yield at levels not previously obtained.

The disclosed production host strains have been engineered to maximize the metabolic efficiency of the pathway by incorporating various deletion mutations that prevent the diversion of carbon to non-productive compounds. It is contemplated that transformations and mutations can be combined so as to control particular enzyme activities for the enhancement of 1,3-propanediol production. Thus, it is within the scope of the present invention to anticipate modifications of a whole cell catalyst which lead to an increased production of 1,3-propanediol.

Terms And Definitions

The invention can be more fully understood with reference to the following terms and definitions used in the claims and specifications.

Genes that are deleted:

The terms "NADH dehydrogenase II", "NDH II" and "Ndh" refer to the type II NADH dehydrogenase, a protein that catalyzed the conversion of ubiquinone-8 + NADH + H^+ to ubiquinol-8 + NAD^+. Typical of NADH dehydrogenase II is EC 1.6.99.3. NADH dehydrogenase II is encoded by *ndh* in *E. coli*.

The terms "aerobic respiration control protein" and "ArcA" refer to a global regulatory protein. The aerobic respiration control protein is encoded by *arcA* in *E. coli*.

The terms "phosphogluconate dehydratase" and "Edd" refer to a protein that catalyzed the conversion of 6-phospho-gluconate to 2-keto-3-deoxy-6-phospho-gluconate + H_2O. Typical of phosphogluconate dehydratase is EC 4.2.1.12. Phosphogluconate dehydratase is encoded by *edd* in *E. coli*.

The terms "phosphocarrier protein HPr" and "PtsH" refer to the phosphocarrier protein encoded by *ptsH* in *E. coli*. The terms "phosphoenolpyruvate-protein phosphotransferase" and "PtsI" refer to the

phosphotransferase, EC 2.7.3.9, encoded by *ptsI* in *E. coli*. The terms "PTS system", "glucose-specific IIA component", and "Crr" refer to EC 2.7.1.69, encoded by *crr* in *E. coli*. PtsH, PtsI, and Crr comprise the PTS system.

The term "phosphoenolpyruvate-sugar phosphotransferase system", "PTS system", or "PTS" refers to the phosphoenolpyruvate-dependent sugar uptake system.

The terms "methylglyoxal synthase" and "MgsA" refer to a protein that catalyzed the conversion of dihydroxy-acetone-phosphate to methyl-glyoxal + phosphate. Typical of methylglyoxal synthase is EC 4.2.3.3. Methylglyoxal synthase is encoded by *mgsA* in *E. coli*.

The terms "aldehyde dehydrogenase A" and "AldA" refer to a protein that catalyzed the conversion of H_2O + NAD^+ + aldehyde to NADH + alcohol. Typical of aldehyde dehydrogenase A is EC 1.2.1.22. Aldehyde dehydrogenase A is encoded by *aldA* in *E. coli*.

The terms "aldehyde dehydrogenase B" and "AldB" refer to a protein that catalyzed the conversion of H_2O + NAD^+ + aldehyde to NADH + alcohol. Typical of aldehyde dehydrogenase B is EC 1.2.1.22. Aldehyde dehydrogenase B is encoded by *aldB* in *E. coli*.

Genes whose expression has been modified:

The terms "galactose-proton symporter" and "GalP" refer to a protein that catalyses the transport of a sugar and a proton from the periplasm to the cytoplasm. D-glucose is a preferred substrate for GalP. Galactose-proton symporter is encoded by *galP* in *E. coli*.

The terms "glucokinase" and "Glk" refer to a protein that catalyses the conversion of D-glucose + ATP to glucose-6-phosphate + ADP. Typical of glucokinase is EC 2.7.1.2. Glucokinase is encoded by *glk* in *E. coli*.

The terms "glyceraldehyde 3-phosphate dehydrogenase" and "GapA" refer to a protein that catalyses the conversion of glyceraldehyde-3-phosphate + phosphate + NAD^+ to 3-phospho-D-glyceroyl-phosphate + NADH + H^+. Typical of glyceraldehyde 3-phosphate dehydrogenase is EC 1.2.1.12. Glyceraldehyde 3-phosphate dehydrogenase is encoded by *gapA* in *E. coli*.

The terms "phosphoenolpyruvate carboxylase" and "Ppc" refer to a protein that catalyses the conversion of phosphoenolpyruvate + H_2O + CO_2 to phosphate + oxaloacetic acid. Typical of phosphoenolpyruvate

carboxylase is EC 4.1.1.31. Phosphoenolpyruvate carboxylase is encoded by *ppc* in *E. coli*.

The term "YciK" refers to a putative enzyme encoded by *yciK* which is translationally coupled to *btuR*, the gene encoding Cob(I)alamin adenosyltransferase in *Escherichia coli*.

The term "cob(I)alamin adenosyltransferase" refers to an enzyme responsible for the transfer of a deoxyadenosyl moiety from ATP to the reduced corrinoid. Typical of cob(I)alamin adenosyltransferase is EC 2.5.1.17. Cob(I)alamin adenosyltransferase is encoded by the gene "*btuR*" (GenBank M21528) in *Escherichia coli*, "*cobA*" (GenBank L08890) in *Salmonella typhimurium*, and "*cobO*" (GenBank M62866) in *Pseudomonas denitrificans*.

Additional definitions:

The term "short 1.20 GI promoter" refers to SEQ ID NO:31. The term "short 1.5 GI promoter" refers to SEQ ID NO:28. The terms "short 1.6 GI promoter" and "short wild-type promoter" are used interchangeably and refer to SEQ ID NO:32.

The terms "glycerol-3-phosphate dehydrogenase" and "G3PDH" refer to a polypeptide responsible for an enzyme activity that catalyzes the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). *In vivo* G3PDH may be NADH; NADPH; or FAD-dependent. When specifically referring to a cofactor specific glycerol-3-phosphate dehydrogenase, the terms "NADH-dependent glycerol-3-phosphate dehydrogenase", "NADPH-dependent glycerol-3-phosphate dehydrogenase" and "FAD-dependent glycerol-3-phosphate dehydrogenase" will be used. As it is generally the case that NADH-dependent and NADPH-dependent glycerol-3-phosphate dehydrogenases are able to use NADH and NADPH interchangeably (for example by the gene encoded by *gpsA*), the terms NADH-dependent and NADPH-dependent glycerol-3-phosphate dehydrogenase will be used interchangeably. The NADH-dependent enzyme (EC 1.1.1.8) is encoded, for example, by several genes including GPD1 (GenBank Z74071x2), or GPD2 (GenBank Z35169x1), or GPD3 (GenBank G984182), or DAR1 (GenBank Z74071x2). The NADPH-dependent enzyme (EC 1.1.1.94) is encoded by *gpsA* (GenBank U321643, (cds 197911-196892) G466746 and L45246). The FAD-dependent enzyme (EC 1.1.99.5) is encoded by GUT2 (GenBank Z47047x23), or *glpD* (GenBank G147838), or *glpABC*

(GenBank M20938) (see WO 9928480 and references therein, which are herein incorporated by reference).

The terms "glycerol-3-phosphatase", "sn-glycerol-3-phosphatase", or "d,l-glycerol phosphatase", and "G3P phosphatase" refer to a polypeptide responsible for an enzyme activity that catalyzes the conversion of glycerol-3-phosphate and water to glycerol and inorganic phosphate. G3P phosphatase is encoded, for example, by GPP1 (GenBank Z47047x125), or GPP2 (GenBank U18813x11) (see WO 9928480 and references therein, which are herein incorporated by reference).

The term "glycerol kinase" refers to a polypeptide responsible for an enzyme activity that catalyzes the conversion of glycerol and ATP to glycerol-3-phosphate and ADP. The high-energy phosphate donor ATP may be replaced by physiological substitutes (e.g., phosphoenolpyruvate). Glycerol kinase is encoded, for example, by GUT1 (GenBank U11583x19) and *glpK* (GenBank L19201) (see WO 9928480 and references therein, which are herein incorporated by reference).

The term "glycerol dehydrogenase" refers to a polypeptide responsible for an enzyme activity that catalyzes the conversion of glycerol to dihydroxyacetone (E.C. 1.1.1.6) or glycerol to glyceraldehyde (E.C. 1.1.1.72). A polypeptide responsible for an enzyme activity that catalyzes the conversion of glycerol to dihydroxyacetone is also referred to as a "dihydroxyacetone reductase". Glycerol dehydrogenase may be dependent upon NADH (E.C. 1.1.1.6), NADPH (E.C. 1.1.1.72), or other cofactors (e.g., E.C. 1.1.99.22). A NADH-dependent glycerol dehydrogenase is encoded, for example, by *gldA* (GenBank U00006) (see WO 9928480 and references therein, which are herein incorporated by reference).

The term "dehydratase enzyme" or "dehydratase" will refer to any enzyme activity that catalyzes the conversion of a glycerol molecule to the product 3-hydroxypropionaldehyde. For the purposes of the present invention the dehydratase enzymes include a glycerol dehydratase (E.C. 4.2.1.30) and a diol dehydratase (E.C. 4.2.1.28) having preferred substrates of glycerol and 1,2-propanediol, respectively. Genes for dehydratase enzymes have been identified *in Klebsiella pneumoniae*, *Citrobacter freundii*, *Clostridium pasteurianum*, *Salmonella typhimurium*, and *Klebsiella oxytoca*. In each case, the dehydratase is composed of

three subunits: the large or "α" subunit, the medium or "β" subunit, and the small or "γ" subunit. Due to the wide variation in gene nomenclature used in the literature, a comparative chart is given in Table 1 to facilitate identification. The genes are also described in, for example, Daniel *et al.* (*FEMS Microbiol. Rev. 22*, 553 (1999)) and Toraya and Mori (*J. Biol. Chem. 274*, 3372 (1999)). Referring to Table 1, genes encoding the large or "α" subunit of glycerol dehydratase include *dhaB1*, *gldA* and *dhaB*; genes encoding the medium or "β" subunit include *dhaB2*, *gldB*, and *dhaC*; genes encoding the small or "γ" subunit include *dhaB3*, *gldC*, and *dhaE*. Also referring to Table 1, genes encoding the large or "α" subunit of diol dehydratase include *pduC* and *pddA*; genes encoding the medium or "β" subunit include *pduD* and *pddB*; genes encoding the small or "γ" subunit include *pduE* and *pddC*.

Table 1: Comparative chart of gene names and GenBank references for dehydratase and dehydratase linked functions.

GENE FUNCTION:	regulatory		unknown		reactivation		1,3-PD dehydrogenase		unknown	
ORGANISM (GenBank Reference)	gene	base pairs	gene	base pairs	Geme	base pairs	gene	base pairs	gene	base pairs
K. pneumoniae (SEQ ID NO:1)	*dhaR*	2209-4134	*orfW*	4112-4642	*OrfX*	4643-4996	*dhaT*	5017-6108	*orfY*	6202-6630
K. pneumoniae (U30903)			*orf2c*	7116-7646	*orf2b*	6762-7115	*dhaT*	5578-6741	*orf2a*	5125-5556
K. pneumoniae (U60992)					*GdrB*					
C. freundii (U09771)	*dhaR*	3746-5671	*orfW*	5649-6179	*OrfX*	6180-6533	*dhaT*	6550-7713	*orfY*	7736-8164
C. pasteurianum (AF051373)										
C. pasteurianum (AF006034)			*orfW*	210-731	*OrfX*	1-196	*dhaT*	1232-2389	*orfY*	746-1177
S. typhimurium (AF026270)					*PduH*	8274-8645				
K. oxytoca (AF017781)					*DdrB*	2063-2440				
K. oxytoca (AF051373)										

GENE FUNCTION:

	dehydratase, α		dehydratase, β		dehydratase, γ		reactivation	
	gene	base pairs	gene	base pairs	Gene	base pairs	gene	base pairs
ORGANISM (GenBank Reference)								
K. pneumoniae (SEQ ID NO:1)	*dhaB1*	7044-8711	*dhaB2*	8724-9308	*dhaB3*	9311-9736	*orfZ*	9749-11572
K. pneumoniae (U30903)	*dhaB1*	3047-4714	*dhaB2*	2450-2890	*dhaB3*	2022-2447	*dhaB4*	186-2009
K. pneumoniae (U60992)	*gldA*	121-1788	*gldB*	1801-2385	*GldC*	2388-2813	*gdrA*	
C. freundii (U09771)	*dhaB*	8556-10223	*dhaC*	10235-10819	*DhaE*	10822-11250	*orfZ*	11261-13072
C. pasteurianum (AF051373)	*dhaB*	84-1748	*dhaC*	1779-2318	*DhaE*	2333-2773	*orfZ*	2790-4598
C. pasteurianum (AF006034)								
S. typhimurium (AF026270)	*pduC*	3557-5221	*pduD*	5232-5906	*PduE*	5921-6442	*pduG*	6452-8284
K. oxytoca (AF017781)							*ddrA*	241-2073
K. oxytoca (AF051373)	*pddA*	121-1785	*pddB*	1796-2470	*PddC*	2485-3006		

Glycerol and diol dehydratases are subject to mechanism-based suicide inactivation by glycerol and some other substrates (Daniel *et al.*, *FEMS Microbiol. Rev. 22*, 553 (1999)). The term "dehydratase reactivation factor" refers to those proteins responsible for reactivating the dehydratase activity. The terms "dehydratase reactivating activity", "reactivating the dehydratase activity" or "regenerating the dehydratase activity" refers to the phenomenon of converting a dehydratase not capable of catalysis of a substrate to one capable of catalysis of a substrate or to the phenomenon of inhibiting the inactivation of a dehydratase or the phenomenon of extending the useful half-life of the dehydratase enzyme *in vivo*. Two proteins have been identified as being involved as the dehydratase reactivation factor (see WO 9821341 (US 6013494) and references therein, which are herein incorporated by reference; Daniel *et al.*, *supra*; Toraya and Mori, *J. Biol. Chem. 274*, 3372 (1999); and Tobimatsu *et al.*, *J. Bacteriol. 181*, 4110 (1999)). Referring to Table 1, genes encoding one of the proteins include *orfZ*, *dhaB4*, *gdrA*, *pduG* and *ddrA*. Also referring to Table 1, genes encoding the second of the two proteins include *orfX*, *orf2b*, *gdrB*, *pduH* and *ddrB*.

The terms "1,3-propanediol oxidoreductase", "1,3-propanediol dehydrogenase" or "DhaT" refer to the polypeptide(s) responsible for an enzyme activity that is capable of catalyzing the interconversion of 3-HPA and 1,3-propanediol provided the gene(s) encoding such activity is found to be physically or transcriptionally linked to a dehydratase enzyme in its natural (i.e., wild type) setting; for example, the gene is found within a *dha* regulon as is the case with *dhaT* from *Klebsiella pneumoniae*. Referring to Table 1, genes encoding a 1,3-propanediol oxidoreductase include *dhaT* from *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Clostridium pasteurianum*. Each of these genes encode a polypeptide belonging to the family of type III alcohol dehydrogenases, exhibits a conserved iron-binding motif, and has a preference for the NAD^+/NADH linked interconversion of 3-HPA and 1,3-propanediol (Johnson and Lin, *J. Bacteriol. 169*, 2050 (1987); Daniel *et al.*, *J. Bacteriol. 177*, 2151 (1995); and Leurs *et al.*, *FEMS Microbiol. Lett. 154*, 337 (1997)). Enzymes with similar physical properties have been isolated from *Lactobacillus brevis* and *Lactobacillus buchneri* (Veiga da Dunha and Foster, *Appl. Environ. Microbiol. 58*, 2005 (1992)).

The term "*dha* regulon" refers to a set of associated genes or open reading frames encoding various biological activities, including but not limited to a dehydratase activity, a reactivation activity, and a 1,3-propanediol oxidoreductase. Typically a dha regulon comprises the open reading frames *dhaR*, *orfY*, *dhaT*, *orfX*, *orfW*, *dhaB1*, *dhaB2*, *dhaB3*, and *orfZ* as described herein.

The term "non-specific catalytic activity" refers to the polypeptide(s) responsible for an enzyme activity that is sufficient to catalyze the interconversion of 3-HPA and 1,3-propanediol and specifically excludes 1,3-propanediol oxidoreductase(s). Typically these enzymes are alcohol dehydrogenases. Such enzymes may utilize cofactors other than NAD^+/NADH, including but not limited to flavins such as FAD or FMN. A gene for a non-specific alcohol dehydrogenase (yqhD) is found, for example, to be endogenously encoded and functionally expressed within *E. coli* K_{12} strains.

The terms "function" or "enzyme function" refer to the catalytic activity of an enzyme in altering the energy required to perform a specific chemical reaction. It is understood that such an activity may apply to a reaction in equilibrium where the production of either product or substrate may be accomplished under suitable conditions.

The terms "polypeptide" and "protein" are used interchangeably.

The terms "carbon substrate" and "carbon source" refer to a carbon source capable of being metabolized by host microorganisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof.

The terms "host cell" or "host microorganism" refer to a microorganism capable of receiving foreign or heterologous genes and of expressing those genes to produce an active gene product.

The terms "foreign gene", "foreign DNA", "heterologous gene" and "heterologous DNA" refer to genetic material native to one organism that has been placed within a host microorganism by various means. The gene of interest may be a naturally occurring gene, a mutated gene, or a synthetic gene.

The terms "transformation" and "transfection" refer to the acquisition of new genes in a cell after the incorporation of nucleic acid. The acquired genes may be integrated into chromosomal DNA or

introduced as extrachromosomal replicating sequences. The term "transformant" refers to the product of a transformation.

The term "genetically altered" refers to the process of changing hereditary material by transformation or mutation.

The terms "recombinant microorganism" and "transformed host" refer to any microorganism having been transformed with heterologous or foreign genes or extra copies of homologous genes. The recombinant microorganisms of the present invention express foreign genes encoding glycerol-3-phosphate dehydrogenase (GPD1), glycerol-3-phosphatase (GPP2), glycerol dehydratase (*dhaB1, dhaB2 and dhaB3*), dehydratase reactivation factor (*orfZ* and *orfX*), and optionally 1,3-propanediol oxidoreductase (*dhaT*) for the production of 1,3-propanediol from suitable carbon substrates. A preferred embodiment is an *E. coli* transformed with these genes but lacking a functional *dhaT*. A host microorganism, other than *E. coli*, may also be transformed to contain the disclosed genes and the gene for the non-specific catalytic activity for the interconversion of 3-HPA and 1,3-propanediol, specifically excluding 1,3-propanediol oxidoreductase(s) (*dhaT*).

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. The terms "native" and "wild-type" refer to a gene as found in nature with its own regulatory sequences.

The terms "encoding" and "coding" refer to the process by which a gene, through the mechanisms of transcription and translation, produces an amino acid sequence. It is understood that the process of encoding a specific amino acid sequence includes DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the invention encompasses more than the specific exemplary sequences.

The term "isolated" refers to a protein or DNA sequence that is removed from at least one component with which it is naturally associated.

An "isolated nucleic acid molecule" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid molecule in

the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

"Substantially similar" refers to nucleic acid molecules wherein changes in one or more nucleotide bases result in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. "Substantially similar" also refers to nucleic acid molecules wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid molecule to mediate alteration of gene expression by antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid molecules of the instant invention (such as deletion or insertion of one or more nucleotide bases) that do not substantially affect the functional properties of the resulting transcript vis-à-vis the ability to mediate alteration of gene expression by antisense or co-suppression technology or alteration of the functional properties of the resulting protein molecule. The invention encompasses more than the specific exemplary sequences.

For example, it is well known in the art that alterations in a gene which result in the production of a chemically equivalent amino acid at a given site, but do not effect the functional properties of the encoded protein are common. For the purposes of the present invention substitutions are defined as exchanges within one of the following five groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: His, Arg, Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys); and
5. Large aromatic residues: Phe, Tyr, Trp.

Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue (such as glycine) or a more hydrophobic residue (such as valine, leucine, or isoleucine). Similarly, changes which result in substitution of one negatively charged residue for another (such as aspartic acid for glutamic acid) or one positively charged residue for another (such as

lysine for arginine) can also be expected to produce a functionally equivalent product.

In many cases, nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein.

Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, the skilled artisan recognizes that substantially similar sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65 °C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS), with the sequences exemplified herein. Preferred substantially similar nucleic acid fragments of the instant invention are those nucleic acid fragments whose DNA sequences are at least 80% identical to the DNA sequence of the nucleic acid fragments reported herein. More preferred nucleic acid fragments are at least 90% identical to the DNA sequence of the nucleic acid fragments reported herein. Most preferred are nucleic acid fragments that are at least 95% identical to the DNA sequence of the nucleic acid fragments reported herein.

A nucleic acid fragment is "hybridizable" to another nucleic acid fragment, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid fragment can anneal to the other nucleic acid fragment under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein (entirely incorporated herein by reference). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T*m* of 55 °, can be used, e.g., 5X SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5X SSC, 0.5% SDS. Moderate stringency hybridization conditions correspond to a higher T*m*, e.g., 40% formamide, with 5X or 6X SSC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are

possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T*m* for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T*m*) of nucleic acid hybridization decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T*m* have been derived (see Sambrook *et al.*, *supra*, 9.50-9.51). For hybridization with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook *et al.*, *supra*, 11.7-11.8). In one embodiment the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferable a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

A "substantial portion" refers to an amino acid or nucleotide sequence which comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to afford putative identification of that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul *et al.*, *J. Mol.Biol.* 215:403-410 (1993); see also www.ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., in situ hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid molecule comprising the primers.

Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to afford specific identification and/or isolation of a nucleic acid molecule comprising the sequence. The instant specification teaches partial or complete amino acid and nucleotide sequences encoding one or more particular proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for the purpose known to those skilled in the art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

The term "complementary" describes the relationship between nucleotide bases that are capable to hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. Accordingly, the instant invention also includes isolated nucleic acid molecules that are complementary to the complete sequences as reported in the accompanying Sequence Listing as well as those substantially similar nucleic acid sequences.

The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: *Computational Molecular Biology*; Lesk, A. M., Ed.; Oxford University Press: New York, 1988; *Biocomputing: Informatics and Genome Projects*; Smith, D. W., Ed.; Academic Press: New York, 1993; *Computer Analysis of Sequence Data, Part I*; Griffin, A. M. and Griffin, H. G., Eds.; Humana Press: New Jersey, 1994; *Sequence Analysis in Molecular Biology*; von Heinje, G., Ed.; Academic Press: New York, 1987; and *Sequence Analysis Primer*; Gribskov, M. and Devereux, J., Eds.; Stockton Press: New York, 1991. Preferred methods to determine identity are designed to give the largest match between the sequences tested.

Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to

determine identity and similarity between two sequences include, but are not limited to, the GCG Pileup program found in the GCG program package, using the Needleman and Wunsch algorithm with their standard default values of gap creation penalty=12 and gap extension penalty=4 (Devereux *et al.*, *Nucleic Acids Res.* 12:387-395 (1984)), BLASTP, BLASTN, and FASTA (Pearson *et al.*, *Proc. Natl. Acad. Sci. USA* 85:2444-2448 (1988). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul *et al.*, Natl. Cent. Biotechnol. Inf., Natl. Library Med. (NCBI NLM) NIH, Bethesda, Md. 20894; Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990); Altschul *et al.*, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402 (1997)). Another preferred method to determine percent identity, is by the method of DNASTAR protein alignment protocol using the Jotun-Hein algorithm (Hein *et al.*, *Methods Enzymol.* 183:626-645 (1990)). Default parameters for the Jotun-Hein method for alignments are: for multiple alignments, gap penalty=11, gap length penalty=3; for pairwise alignments ktuple=6. As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, by a polypeptide having an amino acid sequence having at least, for example, 95% identity to a reference amino acid sequence is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per

each 100 amino acids of the reference amino acid. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

The term "homologous" refers to a protein or polypeptide native or naturally occurring in a given host cell. The invention includes microorganisms producing homologous proteins via recombinant DNA technology.

The term "percent homology" refers to the extent of amino acid sequence identity between ppolypeptides. When a first amino acid sequence is identical to a ssecond amino acid sequence, then the first and second amino acid sequences exhibit 100% homology. The homology between any two polypeptides is a direct function of the total number of matching amino acids at a given position in either sequence, e.g., if half of the total number of amino acids in either of the two sequences are the same then the two sequences are said to exhibit 50% homology.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alteration in the gene sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given

site, are contemplated. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity in the encoded products. Moreover, the skilled artisan recognizes that sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65 °C), with the sequences exemplified herein.

The term "expression" refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product.

The terms "plasmid", "vector", and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

Construction of Recombinant Organisms

Recombinant organisms containing the necessary genes that will encode the enzymatic pathway for the conversion of a carbon substrate to 1,3-propanediol may be constructed using techniques well known in the art. Genes encoding glycerol-3-phosphate dehydrogenase (GPD1), glycerol-3-phosphatase (GPP2), glycerol dehydratase (*dhaB1, dhaB2, and dhaB3*), dehydratase reactivation factor (*orfZ* and *orfX*) and 1,3-propanediol oxidoreductase (*dhaT*) were isolated from a native host such as *Klebsiella* or *Saccharomyces* and used to transform host strains such as *E. coli* DH5α, ECL707, AA200, or KLP23.

Isolation of Genes

Methods of obtaining desired genes from a bacterial genome are common and well known in the art of molecular biology. For example, if the sequence of the gene is known, suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes complementary to the desired gene sequence. Once the sequence is isolated, the DNA may be amplified using standard primer directed amplification methods such as polymerase chain reaction (PCR) (US 4,683,202) to obtain amounts of DNA suitable for transformation using appropriate vectors.

Alternatively, cosmid libraries may be created where large segments of genomic DNA (35-45kb) may be packaged into vectors and used to transform appropriate hosts. Cosmid vectors are unique in being able to accommodate large quantities of DNA. Generally cosmid vectors have at least one copy of the *cos* DNA sequence which is needed for packaging and subsequent circularization of the foreign DNA. In addition to the *cos* sequence these vectors will also contain an origin of replication such as ColE1 and drug resistance markers such as a gene resistant to ampicillin or neomycin. Methods of using cosmid vectors for the transformation of suitable bacterial hosts are well described in Sambrook, J. et al., *supra*.

Typically to clone cosmids, foreign DNA is isolated using the appropriate restriction endonucleases and ligated, adjacent to the *cos* region of the cosmid vector using the appropriate ligases. Cosmid vectors containing the linearized foreign DNA are then reacted with a DNA packaging vehicle such as bacteriophage. During the packaging process the *cos* sites are cleaved and the foreign DNA is packaged into the head

portion of the bacterial viral particle. These particles are then used to transfect suitable host cells such as *E. coli*. Once injected into the cell, the foreign DNA circularizes under the influence of the *cos* sticky ends. In this manner large segments of foreign DNA can be introduced and expressed in recombinant host cells.

Isolation And Cloning of Genes Encoding Glycerol Dehydratase (*dhaB1*, *dhaB2*, and *dhaB3*), Dehydratase Reactivating Factors (*orfZ* and *orfX*), and 1,3-propanediol dehydrogenase (*dhaT*)

Cosmid vectors and cosmid transformation methods were used within the context of the present invention to clone large segments of genomic DNA from bacterial genera known to possess genes capable of processing glycerol to 1,3-propanediol. Specifically, genomic DNA from *K. pneumoniae* was isolated by methods well known in the art and digested with the restriction enzyme Sau3A for insertion into a cosmid vector Supercos 1 and packaged using GigapackII packaging extracts. Following construction of the vector *E. coli* XL1-Blue MR cells were transformed with the cosmid DNA. Transformants were screened for the ability to convert glycerol to 1,3-propanediol by growing the cells in the presence of glycerol and analyzing the media for 1,3-propanediol formation.

Two of the 1,3-propanediol positive transformants were analyzed and the cosmids were named pKP1 and pKP2. DNA sequencing revealed extensive homology to the glycerol dehydratase gene from *C. freundii*, demonstrating that these transformants contained DNA encoding the glycerol dehydratase gene. Other 1,3-propanediol positive transformants were analyzed and the cosmids were named pKP4 and pKP5. DNA sequencing revealed that these cosmids carried DNA encoding a diol dehydratase gene.

Although the instant invention utilizes the isolated genes from within a *Klebsiella* cosmid, alternate sources of dehydratase genes and dehydratase reactivation factor genes include, but are not limited to, *Citrobacter*, *Clostridia* and *Salmonella* (see Table 1).

Genes Encoding G3PDH and G3P Phosphatase

The present invention provides genes suitable for the expression of G3PDH and G3P phosphatase activities in a host cell.

Genes encoding G3PDH are known. For example, GPD1 has been isolated from *Saccharomyces* (Wang *et al.*, *supra*). Similarly, G3PDH

activity has also been isolated from *Saccharomyces* encoded by GPD2 (Eriksson *et al.*, *Mol. Microbiol. 17*, 95 (1995)).

For the purposes of the present invention it is contemplated that any gene encoding a polypeptide responsible for NADH-dependent G3PDH activity is suitable wherein that activity is capable of catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). Further, it is contemplated that any gene encoding the amino acid sequence of NADH-dependent G3PDH's corresponding to the genes DAR1, GPD1, GPD2, GPD3, and *gpsA* will be functional in the present invention wherein that amino acid sequence may encompass amino acid substitutions, deletions or additions that do not alter the function of the enzyme. The skilled person will appreciate that genes encoding G3PDH isolated from other sources will also be suitable for use in the present invention. Genes encoding G3P phosphatase are known. For example, GPP2 has been isolated from *Saccharomyces cerevisiae* (Norbeck *et al.*, *J. Biol. Chem. 271*, 13875 (1996)).

For the purposes of the present invention, any gene encoding a G3P phosphatase activity is suitable for use in the method wherein that activity is capable of catalyzing the conversion of glycerol-3-phosphate plus H_2O to glycerol plus inorganic phosphate. Further, any gene encoding the amino acid sequence of G3P phosphatase corresponding to the genes GPP2 and GPP1 will be functional in the present invention including any amino acid sequence that encompasses amino acid substitutions, deletions or additions that do not alter the function of the G3P phosphatase enzyme. The skilled person will appreciate that genes encoding G3P phosphatase isolated from other sources will also be suitable for use in the present invention.

Host Cells

Suitable host cells for the recombinant production of 1,3-propanediol may be either prokaryotic or eukaryotic and will be limited only by the host cell ability to express the active enzymes for the 1,3-propanediol pathway. Suitable host cells will be microorganisms such as *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces*, and *Pseudomonas*. Preferred in the

present invention are *Escherichia coli*, *Escherichia blattae*, *Klebsiella*, *Citrobacter,* and *Aerobacter*. Most preferred is *E. coli* (KLP23 (WO 2001012833 A2), RJ8.n (ATCC PTA-4216), *E. coli*: FMP'::Km (ATCC PTA4732), MG 1655 (ATCC 700926)).

Vectors and Expression Cassettes

The present invention provides a variety of vectors and transformation and expression cassettes suitable for the cloning, transformation and expression of G3PDH, G3P phosphatase, dehydratase, and dehydratase reactivation factor into a suitable host cell. Suitable vectors will be those which are compatible with the microorganism employed. Suitable vectors can be derived, for example, from a bacterium, a virus (such as bacteriophage T7 or a M-13 derived phage), a cosmid, a yeast or a plant. Protocols for obtaining and using such vectors are known to those in the art (Sambrook *et al.*, *supra*).

Initiation control regions, or promoters, which are useful to drive expression of the G3PDH and G3P phosphatase genes (DAR1 and GPP2, respectively) in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genes is suitable for the present invention including but not limited to CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, and TPI (useful for expression in *Saccharomyces*); AOX1 (useful for expression in *Pichia*); and lac, trp, λP_L, λP_R, T7, tac, and trc (useful for expression in *E. coli*).

Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary; however, it is most preferred if included.

For effective expression of the instant enzymes, DNA encoding the enzymes are linked operably through initiation codons to selected expression control regions such that expression results in the formation of the appropriate messenger RNA.

Particularly useful in the present invention are the vectors pSYCO101, pSYCO103, pSYCO106, and pSYCO109. The essential elements are derived from the *dha* regulon isolated from *Klebsiella pneumoniae* and from *Saccharomyces cerevisiae*. Each contains the open reading frames *dhaB1, dhaB2, dhaB3*, dhaX, orfX, DAR1, and GPP2 arranged in three separate operons, nucleotide sequences of which are given in SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, and SEQ ID

NO:68, respectively. The differences between the vectors are illustrated in the chart below [the prefix "p-" indicates a promoter; the open reading frames contained within each "( )" represent the composition of an operon]:

pSYCO101 (SEQ ID NO:65):

p-trc (Dar1_GPP2) in opposite orientation compared to the other 2 pathway operons,

p-1.6 long GI (dhaB1_dhaB2_dhaB3_dhaX), and

p-1.6 long GI (orfY_orfX_orfW).

pSYCO103 (SEQ ID NO:66):

p-trc (Dar1_GPP2) same orientation compared to the other 2 pathway operons,

p-1.5 long GI (dhaB1_dhaB2_dhaB3_dhaX), and

p-1.5 long GI (orfY_orfX_orfW).

pSYCO106 (SEQ ID NO:67):

p-trc (Dar1_GPP2) same orientation compared to the other 2 pathway operons,

p-1.6 long GI (dhaB1_dhaB2_dhaB3_dhaX), and

p-1.6 long GI (orfY_orfX_orfW).

pSYCO109 (SEQ ID NO:68):

p-trc (Dar1_GPP2) same orientation compared to the other 2 pathway operons,

p-1.6 long GI (dhaB1_dhaB2_dhaB3_dhaX), and

p-1.6 long GI (orfY_orfX).

Transformation of Suitable Hosts and Expression of Genes for the Production of 1,3-propanediol

Once suitable cassettes are constructed they are used to transform appropriate host cells. Introduction of the cassette containing the genes encoding G3PDH, G3P phosphatase, dehydratase, and dehydratase reactivation factor into the host cell may be accomplished by known procedures such as by transformation (e.g., using calcium-permeabilized cells, electroporation), or by transfection using a recombinant phage virus (Sambrook et al., *supra*).

In the present invention cassettes were used to transform the *E. coli* as fully described in the GENERAL METHODS and EXAMPLES.

Mutants

In addition to the cells exemplified, it is contemplated that the present method will be able to make use of cells having single or multiple mutations specifically designed to enhance the production of 1,3-propanediol. Cells that normally divert a carbon feed stock into non-productive pathways, or that exhibit significant catabolite repression could be mutated to avoid these phenotypic deficiencies. For example, many wild-type cells are subject to catabolite repression from glucose and by-products in the media and it is contemplated that mutant strains of these wild-type organisms, capable of 1,3-propanediol production that are resistant to glucose repression, would be particularly useful in the present invention.

Methods of creating mutants are common and well known in the art. For example, wild-type cells may be exposed to a variety of agents such as radiation or chemical mutagens and then screened for the desired phenotype. When creating mutations through radiation either ultraviolet (UV) or ionizing radiation may be used. Suitable short wave UV wavelengths for genetic mutations will fall within the range of 200 nm to 300 nm where 254 nm is preferred. UV radiation in this wavelength principally causes changes within nucleic acid sequence from guanidine and cytosine to adenine and thymidine. Since all cells have DNA repair mechanisms that would repair most UV induced mutations, agents such as caffeine and other inhibitors may be added to interrupt the repair process and maximize the number of effective mutations. Long wave UV mutations using light in the 300 nm to 400 nm range are also possible but are generally not as effective as the short wave UV light unless used in conjunction with various activators such as psoralen dyes that interact with the DNA.

Mutagenesis with chemical agents is also effective for generating mutants and commonly used substances include chemicals that affect nonreplicating DNA such as HNO_2 and NH_2OH, as well as agents that affect replicating DNA such as acridine dyes, notable for causing frameshift mutations. Specific methods for creating mutants using radiation or chemical agents are well documented in the art. See, for example, Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc.,

Sunderland, MA., or Deshpande, Mukund V., *Appl. Biochem. Biotechnol. 36*, 227 (1992), herein incorporated by reference.

After mutagenesis has occurred, mutants having the desired phenotype may be selected by a variety of methods. Random screening is most common where the mutagenized cells are selected for the ability to produce the desired product or intermediate. Alternatively, selective isolation of mutants can be performed by growing a mutagenized population on selective media where only resistant colonies can develop. Methods of mutant selection are highly developed and well known in the art of industrial microbiology. See for example Brock, *Supra*; DeMancilha *et al., Food Chem. 14*, 313 (1984).

In addition to the methods for creating mutants described above, selected genes involved in converting carbon substrate to 1,3-propanediol may be up-regulated or down-regulated by a variety of methods which are known to those skilled in the art. It is well understood that up-regulation or down-regulation of a gene refers to an alteration in the activity of the protein encoded by that gene relative to a control level of activity, for example, by the activity of the protein encoded by the corresponding (or non-altered) wild-type gene.

Up-Regulation:

Specific genes involved in an enzyme pathway may be up-regulated to increase the activity of their encoded function(s). For example, additional copies of selected genes may be introduced into the host cell on multicopy plasmids such as pBR322. Such genes may also be integrated into the chromosome with appropriate regulatory sequences that result in increased activity of their encoded functions. The target genes may be modified so as to be under the control of non-native promoters or altered native promoters. Endogenous promoters can be altered *in vivo* by mutation, deletion, and/or substitution.

Down-Regulation:

Alternatively, it may be useful to reduce or eliminate the expression of certain genes relative to a given activity level. For the purposes of this invention, it is useful to distinguish between reduction and elimination. The terms "down regulation" and "down-regulating" of a gene refers to a reduction, but not a total elimination, of the activity of the encoded protein. Methods of down-regulating and disrupting genes are known to those of skill in the art.

Down-regulation can occur by deletion, insertion, or alteration of coding regions and/or regulatory (promoter) regions. Specific down regulations may be obtained by random mutation followed by screening or selection, or, where the gene sequence is known, by direct intervention by molecular biology methods known to those skilled in the art. A particularly useful, but not exclusive, method to effect down-regulation is to alter promoter strength.

Disruption:

Disruptions of genes may occur, for example, by 1) deleting coding regions and/or regulatory (promoter) regions, 2) inserting exogenous nucleic acid sequences into coding regions and/regulatory (promoter) regions, and 3) altering coding regions and/or regulatory (promoter) regions (for example, by making DNA base pair changes). Such changes would either prevent expression of the protein of interest or result in the expression of a protein that is non-functional. Specific disruptions may be obtained by random mutation followed by screening or selection, or, in cases where the gene sequences in known, specific disruptions may be obtained by direct intervention using molecular biology methods know to those skilled in the art. A particularly useful method is the deletion of significant amounts of coding regions and/or regulatory (promoter) regions.

Methods of altering recombinant protein expression are known to those skilled in the art, and are discussed in part in Baneyx, *Curr. Opinion Biotech.* (1999) 10:411; Ross, et al., *J Bacteriol.* (1998) 180:5375; deHaseth, et al., *J. Bacteriol.* (1998) 180:3019; Smolke and Keasling, *Biotech. And Bioengineeering* (2002) 80:762; Swartz, *Curr. Opinions Biotech.*(2001) 12:195; and Ma, et al., *J. Bacteriol.* (2002) 184:5733.

Alterations in the 1,3-propanediol Production Pathway

Representative Enzyme Pathway. The production of 1,3-propanediol from glucose can be accomplished by the following series of steps. This series is representative of a number of pathways known to those skilled in the art. Glucose is converted in a series of steps by enzymes of the glycolytic pathway to dihydroxyacetone phosphate (DHAP) and 3-phosphoglyceraldehyde (3-PG). Glycerol is then formed by either hydrolysis of DHAP to dihydroxyacetone (DHA) followed by reduction, or reduction of DHAP to glycerol 3-phosphate (G3P) followed by hydrolysis. The hydrolysis step can be catalyzed by any number of cellular phosphatases, which are known to be non-specific with respect to their

substrates, or the activity can be introduced into the host by recombination. The reduction step can be catalyzed by a NAD^+ (or $NADP^+$) linked host enzyme or the activity can be introduced into the host by recombination. It is notable that the *dha* regulon contains a glycerol dehydrogenase (E.C. 1.1.1.6) that catalyzes the reversible reaction of Equation 3.

$$\text{Glycerol} \rightarrow \text{3-HPA} + H_2O \qquad \text{(Equation 1)}$$

$$\text{3-HPA} + NADH + H^+ \rightarrow \text{1,3-Propanediol} + NAD^+ \qquad \text{(Equation 2)}$$

$$\text{Glycerol} + NAD^+ \rightarrow \text{DHA} + NADH + H^+ \qquad \text{(Equation 3)}$$

Glycerol is converted to 1,3-propanediol via the intermediate 3-hydroxy-propionaldehye (3-HPA) as has been described in detail above. The intermediate 3-HPA is produced from glycerol, Equation 1, by a dehydratase enzyme that can be encoded by the host or can be introduced into the host by recombination. This dehydratase can be glycerol dehydratase (E.C. 4.2.1.30), diol dehydratase (E.C. 4.2.1.28) or any other enzyme able to catalyze this transformation. Glycerol dehydratase, but not diol dehydratase, is encoded by the *dha* regulon. 1,3-Propanediol is produced from 3-HPA, Equation 2, by a NAD^+- (or $NADP^+$) linked host enzyme or the activity can be introduced into the host by recombination. This final reaction in the production of 1,3-propanediol can be catalyzed by 1,3-propanediol dehydrogenase (E.C. 1.1.1.202) or other alcohol dehydrogenases.

Mutations and transformations that affect carbon channeling. A variety of mutant microorganisms comprising variations in the 1,3-propanediol production pathway will be useful in the present invention. Mutations which block alternate pathways for intermediates of the 1,3-propanediol production pathway would also be useful to the present invention. For example, the elimination of glycerol kinase prevents glycerol, formed from G3P by the action of G3P phosphatase, from being re-converted to G3P at the expense of ATP. Also, the elimination of glycerol dehydrogenase (for example, *gldA*) prevents glycerol, formed from DHAP by the action of NADH-dependent glycerol-3-phosphate dehydrogenase, from being converted to dihydroxyacetone. Mutations can be directed toward a structural gene so as to impair or improve the activity of an enzymatic activity or can be directed toward a regulatory gene, including promoter

regions and ribosome binding sites, so as to modulate the expression level of an enzymatic activity.

It is thus contemplated that transformations and mutations can be combined so as to control particular enzyme activities for the enhancement of 1,3-propanediol production. Thus, it is within the scope of the present invention to anticipate modifications of a whole cell catalyst which lead to an increased production of 1,3-propanediol.

The present invention utilizes a preferred pathway for the production of 1,3-propanediol from a sugar substrate where the carbon flow moves from glucose to DHAP, G3P, Glycerol, 3-HPA, and finally to 1,3-propanediol. The present production strains have been engineered to maximize the metabolic efficiency of the pathway by incorporating various deletion mutations that prevent the diversion of carbon to non-productive compounds. Glycerol may be diverted from conversion to 3HPA by transformation to either DHA or G3P via glycerol dehydrogenase or glycerol kinase as discussed above. Accordingly, the present production strains contain deletion mutations in the *gldA* and *glpK* genes. Similarly DHAP may be diverted to 3-PG by triosephosphate isomerase, thus the present production microorganism also contains a deletion mutation in this gene. The present method additionally incorporates a dehydratase enzyme for the conversion of glycerol to 3HPA, which functions in concert with the reactivation factor, encoded by *orfX* and *orfZ* of the *dha* regulon. Although conversion of the 3HPA to 1,3-propanediol is typically accomplished via a 1,3-propanediol oxidoreductase, the present method utilizes a non-specific catalytic activity that produces greater titers and yields of the final product, 1,3-propanediol. In such a process, titers of 1,3-propanediol of at least 10 g/L are achieved, where titers of 200 g/L are expected.

Alternatively, an improved process for 1,3-propanediol production may utilize glycerol or dihydroxyacetone as a substrate where the pathway comprises only the last three substrates, glycerol $\rightarrow$ 3HPA $\rightarrow$ 1,3-propanediol. In such a process, the oxidoreductase is again eliminated in favor of the non-specific catalytic activity (expected to be an alcohol dehydrogenase), however the need for deletion mutations are nullified by the energy considerations of adding glycerol to the culture. In such as process titers of 1,3-propanediol of at least 71 g/L are achieved where titers of 200 g/L are expected.

Similarly it is within the scope of the invention to provide mutants of wild-type microorganisms that have been modified by the deletion or mutation of the *dhaT* activity to create improved 1,3-propandiol producers. For example, microorganisms, which naturally contain all the elements of the dha regulon, may be manipulated so as to inactivate the *dhaT* gene encoding the 1,3-propanediol oxidoreductase activity. These microorganisms will be expected to produce higher yields and titers of 1,3-propanediol, mediated by the presence of an endogenous catalytic activity, expected to be an alcohol dehydrogenase. Examples of such microorganisms include but are not limited to *Klebsiella sp.*, *Citrobacter sp.*, and *Clostridium sp.*

Media and Carbon Substrates

Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates may include but are not limited to monosaccharides such as glucose and fructose and oligosaccharides such as lactose or sucrose.

In the present invention, the preferred carbon substrate is glucose.

In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for 1,3-propanediol production. Particular attention is given to Co(II) salts and/or vitamin B_{12} or precursors thereof.

Adenosyl-cobalamin (coenzyme B_{12}) is an essential cofactor for dehydratase activity. Synthesis of coenzyme B_{12} is found in prokaryotes, some of which are able to synthesize the compound *de novo*, for example, *Escherichia blattae*, *Klebsiella* species, *Citrobacter* species, and *Clostridium* species, while others can perform partial reactions. *E. coli*, for example, cannot fabricate the corrin ring structure, but is able to catalyze the conversion of cobinamide to corrinoid and can introduce the 5'-deoxyadenosyl group. Thus, it is known in the art that a coenzyme B_{12} precursor, such as vitamin B_{12}, need be provided in *E. coli* fermentations.

Vitamin B_{12} additions to *E. coli* fermentations may be added continuously, at a constant rate or staged as to coincide with the generation of cell mass, or may be added in single or multiple bolus additions. Preferred ratios of vitamin B_{12} (mg) fed to cell mass (OD550)

are from 0.06 to 0.60. Most preferred ratios of vitamin B_{12} (mg) fed to cell mass (OD550) are from 0.12 to 0.48.

Although vitamin B_{12} is added to the transformed *E. coli* of the present invention it is contemplated that other microorganisms, capable of *de novo* B_{12} biosynthesis will also be suitable production cells and the addition of B_{12} to these microorganisms will be unnecessary.

Culture Conditions:

Typically cells are grown at 35 °C in appropriate media. Preferred growth media in the present invention are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast medium (YM) broth. Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular microorganism will be known by someone skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, e.g., cyclic adenosine 2':3'-monophosphate, may also be incorporated into the reaction media. Similarly, the use of agents known to modulate enzymatic activities (e.g., methyl viologen) that lead to enhancement of 1,3-propanediol production may be used in conjunction with or as an alternative to genetic manipulations.

Suitable pH ranges for the fermentation are between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred as the initial condition.

Reactions may be performed under aerobic or anaerobic conditions where aerobic, anoxic, or anaerobic conditions are preferred based on the requirements of the microorganism.

Fed-batch fermentations may be performed with carbon feed, for example, glucose, limited or excess.

Batch and Continuous Fermentations:

The present process employs a batch method of fermentation. Classical batch fermentation is a closed system where the composition of the media is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the media is inoculated with the desired microorganism or microorganisms and fermentation is permitted to occur adding nothing to the system. Typically, however, "batch" fermentation is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch

systems the metabolite and biomass compositions of the system change constantly up to the time the fermentation is stopped. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of production of end product or intermediate.

A variation on the standard batch system is the Fed-Batch system. Fed-Batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO_2. Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in Brock, *supra*.

Although the present invention is performed in batch mode it is contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to media being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing

the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

It is contemplated that the present invention may be practiced using batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells may be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for 1,3-propanediol production.

Identification and purification of 1,3-propanediol:

Methods for the purification of 1,3-propanediol from fermentation media are known in the art. For example, propanediols can be obtained from cell media by subjecting the reaction mixture to extraction with an organic solvent, distillation, and column chromatography (U.S. 5,356,812). A particularly good organic solvent for this process is cyclohexane (U.S. 5,008,473).

1,3-Propanediol may be identified directly by submitting the media to high pressure liquid chromatography (HPLC) analysis. Preferred in the present invention is a method where fermentation media is analyzed on an analytical ion exchange column using a mobile phase of 0.01N sulfuric acid in an isocratic fashion.

GENERAL METHODS AND MATERIALS

Procedures for phosphorylations, ligations and transformations are well known in the art. Techniques suitable for use in the following examples may be found in Sambrook, J. *et al.*, *supra*.

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, D.C. (1994) or Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA. All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "hr" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL"

means milliliters, "L" means liters, 50 amp is 50 μg/mL ampicillin, and LB-50 amp is Luria-Bertani broth containing 50 μg/mL ampicillin.

Within the tables the following abbreviations are used. "Con." is conversion, "Sel." is selectivity based on carbon, and "nd" is not detected.

Strains and vectors used and constructed in the following examples are listed in the chart below:

Name	Alternate name	Phenotype
FM5		
RJF10		FM5 glpK-
KLP23 WO2001012833 A2		FM5 glpK- gldA-
KLndh81		FM5 glpk- gldA- ndh-
KLpts7		FM5 glpk- gldA- ndh- ptsHIcrr- KmR
KlgalP-trc		FM5 glpk- gldA- ndh- ptsHIcrr- KmR galPp-trc
KLGG		FM5 glpk- gldA- ndh- ptsHIcrr- KmR galPp-trc glkp-trc
KLGG ΔarcA		FM5 glpk- gldA- ndh- ptsHIcrr- KmR galPp-trc glkp-trc arcA-
KLGG ΔarcA Δedd	FMP	FM5 glpk- gldA- ndh- ptsHIcrr- KmR galPp-trc glkp-trc arcA- edd-
Selected FMP	FMP'::Km	FM5 glpk- gldA- ndh- ptsHIcrr- KmR galPp-trc glkp-trc* arcA- edd-
FMP'::Km 1.5gapA		FM5 glpk- gldA- ndh- ptsHIcrr- KmR galPp-trc glkp-trc* arcA- edd- gapAp-1.5
FMP' 1.5 gapA		FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5
FMP' 1.5 gapA ΔmgsA		FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5 mgsA-
FMP' 1.5 gap Δmgs 1.6ppc	Triple	FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5 mgsA- ppcp-1.6
Triple 1.6 btuR		FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5 mgsA- ppcp-1.6 yciK-btuRp-1.6

Name	Alternate name	Phenotype
Triple 1.6 btuR 1.6yqhD		FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5 mgsA-ppcp-1.6 yciK-btuRp-1.6 yqhC- yqhDp-1.6
Triple 1.6 btuR 1.6yqhD ΔackA-pta	Triple Triple	FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5 mgsA-ppcp-1.6 yciK-btuRp-1.6 yqhC- yqhDp-1.6 ackA-pta-
Triple Triple ΔaldA		FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5 mgsA-ppcp-1.6 yciK-btuRp-1.6 yqhC- yqhDp-1.6 ackA-pta- aldA-
Triple Triple ΔaldB		FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5 mgsA-ppcp-1.6 yciK-btuRp-1.6 yqhC- yqhDp-1.6 ackA-pta- aldB-
Triple Triple ΔaldA ΔaldB		FM5 glpk- gldA- ndh- ptsHIcrr- galPp-trc glkp-trc* arcA- edd- gapAp-1.5 mgsA-ppcp-1.6 yciK-btuRp-1.6 yqhC- yqhDp-1.6 ackA-pta- aldA- aldB-

Strains:

KLP23 (WO 2001012833 A2),

RJ8.n (ATCC PTA-4216),

MG 1655 ATCC 700926 (commercially available)

Plasmids:

pAH48 WO 9821340 A1

pDT29 WO 2001012833 A2

pKP32 WO 2001012833 A2

pSYCO101 SEQ ID NO:65.

pSYCO103 SEQ ID NO:66.

pSYCO106 SEQ ID NO:67.

pSYCO109 SEQ ID NO:68.

The plasmids pKD3, pKD4, pKD13 and pKD46, and pCP20 have been described (Datsenko and Wanner, *supra*). The plasmids pLoxCat2 and pJW168 have been described (Palmeros *et al.*, *supra*).

Chromosomal Integration for Gene Knockouts, Promoter Replacements and Introduction of Chromosomal Mutations.

To integrate DNA into a specific region of the chromosome, homology of the inserting DNA to the targeted chromosomal site and a selectable marker are required. It is advantageous if the marker can be easily removed after integration. The *loxP*/Cre recombinase system from P1 phage and the FRT/Flp recombinase system from yeast provide a mechanism to remove the marker. The *loxP* and FRT sites are recognition sites for the Cre and Flp recombinases. Cre and Flp are site specific recombinases, which excise the intervening DNA from the directly repeated recognition sites.

The integration cassette containing homologous arms to the targeted chromosomal site and encoding a selectable marker flanked by *loxP* [Palmeros *et al. Gene* 247, 255–264 (2000)] or FRT [Datsenko and Wanner, *Proc. Natl. Acad. Sci. USA* 97, 6640-6645 (2000)] sites is transformed into target cells harboring pKD46 (Datsenko and Wanner, *supra*). Successful integrants are selected by growth of the cells in the presence of the antibiotic. Subsequently, pKD46 is cured from the cells and the recombinase plasmids are then introduced into the integrants for removal of the antibiotic gene. Strains integrated with a *loxP* cassette are transformed with pJW168 that encodes Cre recombinase (Palmeros *et al, supra*). Strains containing a FRT cassette are transformed with pCP20 that encodes Flp recombinase (Datsenko and Wanner, *supra*). After removal of the integrated marker, the recombinase plasmids are cured from the strain.

P1 *vir* transduction were performed as previously described [Miller, J.H., A short course in bacterial genetics. A laboratory manual and handbook for *Escherchia coli* and related bacteria (1992), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY].

ENZYME ASSAYS

Assays for Glucokinase (Glk) activity:

Glucokinase (Glk) activity was assayed by following the conversion of glucose to glucose-6-phosphate spectrophotometrically at 340 nm by coupling the glucokinase reaction with that of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49). The assay contained 0.5 mM NADP, 5 mM ATP, 5 mM MgCl, and 2 units of glucose-6-phosphate dehydrogenase

in 100 mM phosphate buffer, pH 7.2. Alternative assays may be found in T. E. Barman, Enzyme Handbook (1985), Springer-Verlag, Berlin.

Assay for Glyceraldehyde-3-phosphate dehydrogenase activity:

Assay for Glyceraldehyde-3-phosphate dehydrogenase activity was measured in cell-free extracts by the appearance of NADH. An ultracentrifuged (50,000 x g, 1 h, 4 °C) cell-free supernatant was partially purified using an ion exchange column prior to assay. The assay contained 0.2 mM glyceraldehyde 3-phosphate, 2.5 mM NAD^+, 2 mM EDTA, 5 mM cysteamine, 20 mM potassium phosphate and 40 mM triethanolamine at pH 8.9. Alternative assays may be found in T. E. Barman, *supra*.

Assay for Phosphoenolpyruvate carboxylase (Ppc) activity:

Phosphoenolpyruvate carboxylase (Ppc) activity was measured in cell-free extracts by a coupled assay (Flores and Gancedo, *FEBS Lett.* 412, 531-534 (1997)). This method involved incubating at room temperature a ultracentifuged (50,000 x g, 1 h, 4 °C) cell-free extract sample in a cuvette that contained 0.22 mM NADH, 1.1 mM phosphoenolpyruvate (PEP), 0.25 mM acetyl-CoA, and 6 U of malate dehydrogenase (MDH) in 0.1 M Tris/HCl buffer, pH 8.5, with 11 mM sodium bicarbonate and 11 mM magnesium sulfate, in a total volume of 1.0 mL. A background rate of the reaction of enzyme and NADH was first determined at 340 nm in the absence of PEP. The second substrate, PEP, was subsequently added and the absorbance change over time was further monitored. Ppc activity was defined by subtracting the background rate from the gross rate. Alternative assays may be found in T. E. Barman, *supra*.

Assays for dehydratase enzymes:

Dehydratase activity in cell-free extracts was determined using either glycerol or 1,2-propanediol as substrate. Typically, cell-free extracts were prepared by cell disruption using a french press followed by centrifugation of the cellular debris. The assay, based on the reaction of aldehydes with methylbenzo-2-thiazolone hydrazone, has been described by Forage and Foster (*Biochim. Biophys. Acta 569*, 249 (1979)).

Honda *et al.* (*J. Bacteriol. 143*, 1458 (1980)) disclose an assay that measures the reactivation of dehydratases. Dehydratase activity was determined in toluenized whole cells, with and without ATP, using either glycerol or 1,2-propanediol as substrate. Reactivation was determined by

the ratio of product formation with versus without the ATP addition. Product formation (3-HPA or propionaldehyde when glycerol or 1,2-propanediol is used as substrate, respectively) was measured directly, using HPLC, or indirectly, using the methylbenzo-2-thiazolone hydrazone reagent. Alternatively, product formation was determined by coupling the conversion of the aldehyde to its respective alcohol using a NADH linked alcohol dehydrogenase and monitoring the disappearance of NADH.

Assays for 1,3-propanediol oxidoreductase:

The activity of 1,3-propanediol oxidoreductase, sometimes referred to as 1,3-propanediol dehydrogenase, was determined for cell-free extracts in solution or in slab gels using 1,3-propanediol and NAD^+ as substrates has been described (Johnson and Lin, *J. Bacteriol. 169*, 2050 (1987)). Alternatively, the conversion of 3-HPA and NADH to 1,3-propanediol and NAD^+ was determined by the disappearance of NADH. The slab gel assay has the potential advantage of separating the activity of 1,3-propanediol oxidoreductase (*dhaT*) from that of non-specific alcohol dehydrogenases by virtue of size separation. The native molecular weights of 1,3-propanediol oxidoreductases (*dhaT*) from *Citrobacter frendii*, *Klebsiella pneumoniae*, and *Clostridium pasteurianum* are unusually large, on the order of 330,000 to 440,000 daltons. *Lactobacillus brevis* and *Lactobacillus buchneri* contain dehydratase associated 1,3-propanediol oxidoreductases with properties similar to those of known 1,3-propanediol oxidoreductases (*dhaT*).

Assays for glycerol 3-phosphate dehydrogenase activity:

A procedure was used as modified below from a method published by Bell *et al.* (*J. Biol. Chem. 250*, 7153 (1975)). This method involved incubating a cell-free extract sample in a cuvette that contained 0.2 mM NADH, 2.0 mM dihydroxyacetone phosphate (DHAP), and enzyme in 0.1 M Tris/HCl, pH 7.5 buffer with 5 mM DTT, in a total volume of 1.0 mL at 30 °C. A background rate of the reaction of enzyme and NADH was first determined at 340 nm for at least 3 min. The second substrate, DHAP, was subsequently added and the absorbance change over time was further monitored for at least 3 min. G3PDH activity was defined by subtracting the background rate from the gross rate.

Assay for glycerol-3-phosphatase activity:

The assay for enzyme activity was performed by incubating the extract with an organic phosphate substrate in a bis-Tris or MES and

magnesium buffer, pH 6.5. The substrate used was either l-α-glycerol phosphate, or d,l-α-glycerol phosphate. The final concentrations of the reagents in the assay are: buffer (20 mM, bis-Tris or 50 mM MES); $MgCl_2$ (10 mM); and substrate (20 mM). If the total protein in the sample was low and no visible precipitation occurs with an acid quench, the sample was conveniently assayed in the cuvette. This method involved incubating an enzyme sample in a cuvette that contained 20 mM substrate (50 μL, 200 mM), 50 mM MES, 10 mM $MgCl_2$, pH 6.5 buffer. The final phosphatase assay volume was 0.5 mL. The enzyme-containing sample was added to the reaction mixture; the contents of the cuvette were mixed and then the cuvette was placed in a circulating water bath at T = 37 °C for 5 to 120 min, the length of time depending on whether the phosphatase activity in the enzyme sample ranged from 2 to 0.02 U/mL. The enzymatic reaction was quenched by the addition of the acid molybdate reagent (0.4 mL). After the Fiske SubbaRow reagent (0.1 mL) and distilled water (1.5 mL) were added, the solution was mixed and allowed to develop. After 10 min, to allow full color development, the absorbance of the samples was read at 660 nm using a Cary 219 UV/vis spectrophotometer. The amount of inorganic phosphate released was compared to a standard curve that was prepared by using a stock inorganic phosphate solution (0.65 mM) and preparing 6 standards with final inorganic phosphate concentrations ranging from 0.026 to 0.130 μmol/mL.

Assay for glycerol kinase activity:

An appropriate amount of enzyme, typically a cell-free crude extract, was added to a reaction mixture containing 40 mM ATP, 20 mM $MgSO_4$, 21 mM uniformly ^{13}C labeled glycerol (99%, Cambridge Isotope Laboratories), and 0.1 M Tris-HCl, pH 9 for 75 min at 25 °C. The conversion of glycerol to glycerol 3-phosphate was detected by ^{13}C-NMR (125 MHz): glycerol (63.11 ppm, δ, J = 41 Hz and 72.66 ppm, t, J = 41 Hz); glycerol 3-phosphate (62.93 ppm, δ, J = 41 Hz; 65.31 ppm, br d, J = 43 Hz; and 72.66 ppm, dt, J = 6, 41 Hz).

NADH-linked glycerol dehydrogenase assay:

NADH –linked glycerol dehydrogenase activity (*gldA*) in cell-free extracts from *E. coli* strains was determined after protein separation by non-denaturing polyacrylamide gel electrophoresis. The conversion of glycerol plus NAD^+ to dihydroxyacetone plus NADH was coupled with the

conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a deeply colored formazan, using phenazine methosulfate (PMS) as mediator (Tang *et al.*, *J. Bacteriol. 140*, 182 (1997)).

Electrophoresis was performed in duplicate by standard procedures using native gels (8-16% TG, 1.5 mm, 15 lane gels from Novex, San Diego, CA). Residual glycerol was removed from the gels by washing 3x with 50 mM Tris or potassium carbonate buffer, pH 9 for 10 min. The duplicate gels were developed, with and without glycerol (approximately 0.16 M final concentration), in 15 mL of assay solution containing 50 mM Tris or potassium carbonate, pH 9, 60 mg ammonium sulfate, 75 mg NAD^+, 1.5 mg MTT, and 0.5 mg PMS.

The presence or absence of NADH –linked glycerol dehydrogenase activity in *E. coli* strains (*gldA*) was also determined, following polyacrylamide gel electrophoresis, by reaction with polyclonal antibodies raised to purified *K. pneumoniae* glycerol dehydrogenase (*dhaD*).

Isolation and identification of 1,3-propanediol:

HPLC analysis of fermentation products. The conversion of glucose to 1,3-propanediol was monitored by HPLC. Analyses were performed using standard chromatography. One suitable method utilized a Waters Alliance HPLC system using RI detection. Samples were injected onto a Aminex HPX87H column (7.8 mm x 300 mm, Biorad, Hercules, CA) equipped with a Cation H Refill Cartridge precolumn (4.6 mm x 30 mm, Biorad, Hercules, CA), temperature controlled at 50 °C, using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.4 mL/min. The system was calibrated weekly against standards of known concentration. Typically, the retention times of glucose, glycerol, 1,3-propanediol, and acetic acid were 12.7 min, 19.0 min, 25.2 min, and 21.5 min, respectively.

GC/MS analysis of fermentation methods.

Production of 1,3-propanediol was confirmed by GC/MS. Analyses were performed using standard techniques and materials available to one of skill in the art of GC/MS. One suitable method utilized a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard 5971 Series mass selective detector (EI) and a HP-INNOWax column (30 m length, 0.25 mm i.d., 0.25 micron film thickness). The retention time and mass spectrum of 1,3-propanediol generated were compared to that of authentic 1,3-propanediol (*m/e*: 57, 58).

An alternative method for GC/MS involved derivatization of the sample. To 1.0 mL of sample (e.g., culture supernatant) was added 30 μL of concentrated (70% v/v) perchloric acid. After mixing, the sample was frozen and lyophilized. A 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide:pyridine (300 μL) was added to the lyophilized material, mixed vigorously and placed at 65 °C for one h. The sample was clarified of insoluble material by centrifugation. The resulting liquid partitioned into two phases, the upper of which was used for analysis. The sample was chromatographed on a DB-5 column (48 m, 0.25 mm I.D., 0.25 μm film thickness; from J&W Scientific) and the retention time and mass spectrum of the 1,3-propanediol derivative obtained from culture supernatants were compared to that obtained from authentic standards. The mass spectra of TMS-derivatized 1,3-propanediol contains the characteristic ions of 205, 177, 130 and 115 AMU.

Medium Composition

TM2 medium (TM2) is a base recipe to which carbon source (typically glucose, at 20 g/L or 40 g/L), appropriate antibiotics, and other components are added. TM2 medium contains the following components: K_2HPO_4 (13.6 g/L), KH_2PO4 (13.6 g/L), $MgSO_4 \cdot 7H_2O$ (2 g/L), citric acid monohydrate (2 g/L), ferric ammonium citrate (0.3 g/L), $(NH_4)_2SO_4$ (3.2 g/L), yeast extract (5 g/L), solution of trace elements (1 ml). The pH is adjusted to 6.8. The solution of trace elements contains: citric acid H_2O (4.0 g/L), $MnSO_4 \cdot H_2O$ (3.0 g/L), NaCl (1.0 g/L), $FeSO_4 \cdot 7H_2O$ (0.10 g/L), $CoCl_2 \cdot 6H_2O$ (0.10 g/L), $ZnSO_4 \cdot 7H_2O$ (0.10 g/L), $CuSO_4 \cdot 5H_2O$ (0.010 g/L), H_3BO_3 (0.010 g/L), and $Na_2MoO_4 \cdot 2H_2O$ (0.010 g/L).

TM3 medium (TM3) is identical to TM2 medium except that it contains 0.5 g/L yeast extract.

LB medium (LB) contains 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl. LB plates (or LA) are LB medium + 2% agar. 2YT medium (2YT) contains 10 g/L yeast extract, 16 g/L tryptone, and 10 g/L NaCl. Soy broth with glucose (SBG1%) contains 10 g/L Soytone (Difco), 5 g/L yeast extract, 10 g/L NaCl, and 10 g/L glucose.

Fermentation Protocol (14L)

Shake flask cultures of all strains described (KLP23, RJ8.n, MB 1655) were grown on either 2YT medium or LB containing the appropriate antibiotics as detailed in the examples to make the pre-culture for

inoculation of the fermenters. Cultures were started from either frozen seed vials prepared with 15% glycerol as a cryoprotectant or from a single colony grown on fresh LA plates with 50 mg/L spectinomycin. Cultures started with a frozen vial were grown in 500 mL of the specified medium in a 2L flask; when a single colony was used to start the pre-culture, it was placed in 30 mL of the specified medium in a 250 mL baffled flask. The cultures were incubated at 34 °C and 300 rpm shaking to an OD_{550} of approximately 1.0 AU was reached and used to seed the fermenter. In some cases, a seed fermenter was used to provide a larger pre-culture for inoculating a production fermenter. Seed fermenters were generally identical to production fermenters except that vitamin B_{12} was not added to the seed tank. Details about the procedures for using seed fermenters are described in the pertinent examples.

Seed and production fermenters were prepared with the same medium, containing KH_2PO_4 (7.5 g/L), $MgSO_4 \cdot 7H_2O$ (2 g/L), citric acid monohydrate (2 g/L), ferric ammonium citrate (0.3 g/L), $CaCl_2 \cdot 2H_2O$ (0.2 g/L), sulfuric acid (98%; 1.2 mL/L), Mazu DF204 (0.4 mL/L) as antifoam, yeast extract (5 g/L), solution of trace elements (10 ml/L). The solution of trace elements contains: citric acid$\cdot H_2O$ (4.0 g/L), $MnSO_4 \cdot H_2O$ (3.0 g/L), NaCl (1.0 g/L), $FeSO_4 \cdot 7H_2O$ (0.10 g/L), $CoCl_2 \cdot 6H_2O$ (0.10 g/L), $ZnSO_4 \cdot 7H_2O$ (0.10 g/L), $CuSO_4 \cdot 5H_2O$ (0.010 g/L), H_3BO_3 (0.010 g/L), and $Na_2MoO_4 \cdot 2H_2O$ (0.010 g/L). After sterilization, the pH was adjusted to 6.8 with 20-28% NH_4OH and additions of glucose (to 10-25 g/L from a 60-67% (w/w) solution) and the appropriate antibiotics (see specific examples for details) were made. The fermenter volume after inoculation was 6 liters.

A 14 L stirred tank fermenter was prepared with the medium described above. The temperature was controlled at 34 °C and aqueous ammonia (20-28 weight %) was used to control pH at 6.8. Backpressure was controlled at 0.5 barg and dO control set at 5%. Except for minor excursions, glucose concentration was maintained between 0 g/L and 25 g/L with a 60-67% (w/w) feed. Vitamin B_{12} additions and any other changes to the general procedure described here are noted in the examples.

Molar yield, as either fraction or %, represents (mol glycerol produced + mol 1,3-propanediol produced)/(mol glucose consumed). Weight yield, generally given as %, represents (g 1,3-propanediol produced)/(g glucose consumed).

EXAMPLE 1

CONSTRUCTION OF NADH DEHYDROGENASE II MINUS (Δ*ndh*) *E. COLI* STRAINS FOR THE PRODUCTION OF 1,3-PROPANEDIOL

Construction of KLndh81.

An *ndh* mutation was obtained by interrupting the coding region with a *loxP*511 cassette. The *ndh* gene (for reference, see GenBank, Accession # U00096), with upstream and downstream flanking regions, was PCR amplified from *E. coli* MG1655 and cloned. The *ndh* cassette was digested with *Stu* I, cutting roughly in the middle of the gene, and a *loxP*511-Cat-*loxP*511cassette was cloned into this site with the *cat* gene in the opposite orientation relative to the *ndh* gene. The *loxP*511-Cat-*loxP*511 cassette was obtained from the plasmid pLoxCat27 [SEQ ID NO:1] by digestion with *Spe* I and *Eco*R V, followed by fill-in to generate blunt ends, and gel purification of the 1.1 kb fragment. The *loxP*511 site is a variant of the *loxP* site (Palmeros *et al., supra*). The ndh::Cat cassette was PCR amplified and electroporated into KLP23 competent cells creating strain KLndh81::Cm. The chloramphenicol marker was removed by the Cre recombinase (Palmeros *et al., supra*) leaving 96 bp of interrupting sequence containing one *loxP*511 site. This strain was designated KLndh81. Alternatively, an *ndh* mutation was obtained by interrupting the coding region with a Cat cassette without *loxP* sites to give strain KLNDH413.

Construction of RJ8.n.

A cassette containing *ndh* flanking sequence and *loxP*511-Cat-*loxP*511 from Klndh81::Cm was PCR amplified and cloned into pUni/V5-His TOPO [Invitrogen] to create pAH111. The ndh-*loxP*511-Cat-*loxP*511 cassette from pAH111 was integrated into strain RJ8/pKD46. Recombinant strains were selected for chloramphenicol resistance. Successful integration of the cassette into *ndh* was confirmed by PCR. The chloramphenicol marker was removed by using Cre recombinase (Palmeros *et al., supra*) creating strain RJ8.n.

EXAMPLE 2

COMPARISON OF 1,3-PROPANEDIOL AND GLYCEROL PRODUCTION WITH *E. COLI* STRAINS KLP23/pAH48/pDT29 AND KLP23/pAH48/pKP32

Strain KLP23 was transformed with plasmids pAH48 and pDT29 or pKP32. Production of 1,3-propanediol (and glycerol) was determined in

14 L fermenters as described in General Methods. Pre-cultures for each fermentation were prepared using frozen vials thawed and grown in 500 mL 2YT with 200 mg/L carbenicillin and 50 mg/L spectinomycin. The full contents of the flask were used to inoculate the fermenter. The fermenter was operated at 35° C and a d6 set-point of 10%; all other control parameters are as described in General Methods. The vitamin B_{12} strategy for each fermentation is detailed below.

Fermentation with KLP23/pAH48/pDT29.

In this example, vitamin B_{12} (0.075 g/L, 500 mL) was fed, starting 3 h after inoculation, at a rate of 16 mL/h. A representative fermentation summary of the conversion of glucose to 1,3-propanediol (1,3-PD) using *E. coli* strain KLP23/pAH48/pDT29 is given in Table 2.1. The yield of 1,3-propanediol was 24 wt % (g 1,3-propanediol/g glucose consumed) and a titer of 68 g/L 1,3-propanediol was obtained.

TABLE 2.1

Representative fermentation summary of the conversion of glucose to 1,3-propanediol (1,3-PD) using *E. coli* strain KLP23/pAH48/pDT29

Time (h)	OD550 (AU)	DO (%)	Glucose (g/L)	Glycerol (g/L)	1,3-PD (g/L)
0	0	150	12.9	0.0	0
6	17	80	8.3	3.1	1
12	42	53	2.8	12.5	9
18	98	9	5.7	12.6	32
24	136	11	32.8	12.0	51
30	148	10	12.3	13.3	62
32	152	11	12.5	14.3	65
38	159	11	1.5	17.2	68

Similar results were obtained with an identical vitamin B_{12} feed at twice the concentration or bolus additions of vitamin B_{12} across the time course of the fermentation. The highest titer obtained was 77 g/L.

Improved fermentation with KLP23/pAH48/pKP32.

A representative fermentation summary of the conversion of glucose to 1,3-propanediol (1,3-PD) using *E. coli* strain KLP23/pAH48/pKP32 is given in Table 2.2. Vitamin B_{12} (0.150 g/L, 500 mL) was fed, starting 3 h after inoculation, at a rate of 16 mL/h. After 36 h, approximately 2 L of fermentation broth was purged in order to allow for the continued addition

of glucose feed. The yield of 1,3-propanediol was 26 wt % (g 1,3-propanediol/g glucose consumed) and a titer of 112 g/L 1,3-propanediol was obtained.

TABLE 2.2

Representative fermentation summary of the improved conversion of glucose to 1,3-propanediol (1,3-PD) using *E. coli* strain KLP23/pAH48/pKP32

Time (h)	OD550 (AU)	DO (%)	Glucose (g/L)	Glycerol (g/L)	1,3-PD (g/L)
0	0	148	12.8	0.0	0
6	22	84	6.9	3.3	0
12	34	90	9.7	10.4	7
18	66	43	9.3	5.9	24
24	161	9	0.2	2.5	46
30	200	10	0.2	6.0	67
36	212	10	1.2	9.7	88
42	202	2	0.1	15.5	98
48	197	12	1.2	23.8	112

Similar results were obtained with an identical vitamin B_{12} feed at half the concentration or bolus additions of vitamin B_{12} across the time course of the fermentation. The highest titer obtained was 114 g/L.

EXAMPLE 2A

COMPARISON OF 1,3-PROPANEDIOL AND GLYCEROL PRODUCTION WITH *E. COLI* STRAINS RJ8/pAH48/pDT29 AND RJ8/pAH48/pKP32

RJ8/pAH48pDT29 and RJ8/pAH48/pKP32 pre-cultures were prepared using frozen vials thawed and grown in 500 mL 2YT with 200 mg/L carbenicillin and 50 mg/L spectinomycin. The full contents of the flask were used to inoculate the fermenter. The fermenter was operated at 35° C and a d6 set-point of 10%; all other control parameters are as described in General Methods. RJ8/pAH48/pKP32 is identical to RJ8/pAH48/pDT29 except that *dhaT* is deleted. The vitamin B_{12} strategy for each fermentation is detailed below.

Fermentation with RJ8/pAH48/pDT29.

A representative fermentation summary of the conversion of glucose to 1,3-propanediol (1,3-PD) using *E. coli* strain RJ8/pAH48/pDT29 is given

in Table 2A.1. Vitamin B_{12} was provided as bolus additions of 2, 16 and 16 mg at 2, 8, and 26 h, respectively. The yield of 1,3-propanediol was 35 wt % (g 1,3-propanediol/g glucose consumed) and a titer of 50.1 g/L 1,3-propanediol was obtained.

TABLE 2A.1

Representative fermentation summary of the conversion of glucose to 1,3-propanediol (1,3-PD) using *E. coli* strain RJ8/pAH48/pDT29

Time (h)	OD550 (AU)	DO (%)	Glucose (g/L)	Glycerol (g/L)	1,3-PD (g/L)
0	0	140	10.6	0.1	0.0
6	5	107	11.1	0.5	0.4
10	16	90	8.5	1.7	1.3
14	25	86	1.8	2.4	5.9
19	38	53	3.5	5.9	15.4
25	53	38	0.1	9.2	26.7
31	54	10	4.5	7.4	39.0
37	37	23	17.2	6.0	45.0
43	21	13	9.9	7.7	50.1

Improved fermentation with RJ8/pAH48/pKP32.

A representative fermentation summary of the conversion of glucose to 1,3-propanediol (1,3-PD) using *E. coli* strain RJ8/pAH48/pKP32 is given in Table 2A.2. Vitamin B_{12} was provided as bolus additions of 48 and 16 mg at approximately 26 and 44 hr, respectively. The yield of 1,3-propanediol was 34 wt % (g 1,3-propanediol/g glucose consumed) and a titer of 129 g/L 1,3-propanediol was obtained.

TABLE 2A.2

Representative fermentation summary of the improved conversion of glucose to 1,3-propanediol (1,3-PD) using *E. coli* strain RJ8/pAH48/pKP32.

Time (h)	OD550 (AU)	DO (%)	Glucose (g/L)	Glycerol (g/L)	1,3-PD (g/L)
0	0	150	12.6	0.1	0
6	12	113	6.0	2.6	0
12	24	99	0.0	10.6	0
18	51	76	2.4	28.9	0
24	78	82	2.4	44.2	5
30	114	70	3.8	26.9	33

Time (h)	OD550 (AU)	DO (%)	Glucose (g/L)	Glycerol (g/L)	1,3-PD (g/L)
36	111	72	0.0	20.0	57
42	139	65	0.1	21.9	69
48	157	36	0.1	22.4	79
55	158	25	0.2	21.4	94
64	169	14	0.1	15.8	113
72	169	12	0.1	13.4	119
74	162	14	0.1	14.8	129

1,3-Propanediol and glycerol production with *E. coli* strain RJ8.n/pAH48/pKP32.

Strain RJ8.n was transformed with plasmids pAH48 and pKP32. Production of 1,3-propanediol (and glycerol) was determined in 14 L fermenters as described in General Methods. A thawed frozen vial of RJ8.n/pAH48/pKP32 was transferred to 500 mL LB with 200 mg/L carbenicillin and 50 mg/L spectinomycin to prepare the pre-culture. The culture was transferred to a seed fermenter and grown for 16 h before 1 L of the culture was transferred to the production fermenter. At that time, the OD550 had reached over 50 AU and 30 g/L glycerol had accumulated in the broth. Both the seed and production fermenters were operated at 35 °C and a d6 set-point of 10%; all other control parameters are as described in General Methods.

Vitamin B_{12} was added to the production tank in 16 mg boluses at 12 h, 17.3 h, 22.8 h, and 27.5 h. The final titer was 112.7 g/L 3G and the mass yield was 31.6%.

EXAMPLE 3

CONSTRUCTION AND SHAKE FLASK PERFORMANCE OF AN *E. COLI* STRAIN WITH A DELETION IN THE *arcA* GLOBAL REGULATOR

An *arcA* deletion [for reference, see GenBank, Accession # U00096] was made by replacing 0.6 kb of the coding region with the FRT-CmR-FRT cassette of pKD3. A replacement cassette was amplified with the primer pair SEQ ID NO:2 and SEQ ID NO:3 using pKD3 as the template. The primer SEQ ID NO:2 contains 41 bp of homology to the 5' end of *arcA* and 20 bp of homology to pKD3. Primer SEQ ID NO:3 contains 42 bp of homology to the 3' end of *arcA* and 20 bp of homology to pKD3. The PCR product was gel-purified and electroporated into

MG1655/pKD46 competent cells. Recombinant strains were selected on LB plates with 12.5 mg/L of chloramphenicol. The deletion of the *arcA* gene was confirmed by PCR, using the primer pair SEQ ID NO:4 and SEQ ID NO:5. The wild-type strain gives a 0.7 kb PCR product while the recombinant strain gives a characteristic 1.1 kb PCR product. The strain has been designated MG1655 ΔarcA::Cm. A P1 lysate was prepared and used to move the mutation into the strain KLndh81 to form KLndh81 ΔarcA::Cm.

The KLndh81 ΔarcA::Cm strain and KLndh81 control strain were electrotransformed with plasmid pSYCO101. One colony of each strain was incubated 10 h in LB medium with 50 mg/L spectinomycin. A 200 μL volume of these cultures was then transferred to a 250 mL baffled Erlenmeyer flask containing 10 mL TM2 medium with 40 g/L glucose, 50 mg/L spectinomycin, and 2 mg/L vitamin B_{12}. The flasks were incubated at 300 rpm and 34 °C for 40 hrs. The results in Table 3 show that the *arcA* mutation improves the molar yield of glycerol and 1, 3 propanediol production.

TABLE 3

Glycerol and 1,3-propanediol production in E. coli control and ΔarcA::Cm strains

Strain	Glycerol (g/L)	1,3 Propanediol (g/L)	OD 550nm	Molar Yield (mol/mol)
KLndh81 pSYCO101	5.8	10.7	29.2	0.87
KLndh81 ΔarcA pSYCO101	6.8	11.4	25.9	0.95

EXAMPLE 4

CONSTRUCTION OF PHOSPHOTRANSERASE SYSTEM MINUS (PTS$^-$) *E. COLI* STRAINS WITH TRC PROMOTERS CONTROLLING EXPRESSION LEVELS OF GALACTOSE-PROTON SYMPORTER (*galP*) AND GLUCOKINASE (*glk*)

Construction of a *loxP*-CAT-*loxP*-Trc cassette (pTrCm42).

Linear DNA was obtained from plasmid pTrc99a (Pharmacia) digested with *Hin*dIII and *Nco*I, filled with T4 DNA polymerase, circularized and transformed into *E.coli* TOP-10 (Invitrogen, Carlsbad, CA). Following

selection on Luria-agar plates containing 50 mg/L of carbenicillin, the resulting plasmid (pTrc1) was purified and subjected to restriction enzyme analysis to confirm that the DNA region originally present between *Hin*dIII and *Nco*I was absent.

The unique *Bsp*M1 site in pTrc1 (upstream of the –35 region of the *trc* promoter) was used to insert a cassette containing a chloramphenicol resistance gene (CAT) flanked by *loxP* sites. Linear DNA was obtained from pTrc1 digested with *Bsp*M1, gel-purified using a QIAquick gel extraction kit (QIAGEN), filled in with T4 DNA polymerase, and ligated to a *loxP*-Cat-*loxP* cassette. The *loxP*-Cat-*loxP* cassette was obtained from the plasmid pLoxCat1, see SEQ ID NO:6, by digestion with *Stu*1 and *Bam*H1. pLoxCat1 is similar to pLoxCat2 (Palmeros *et al, supra*]. The ligation mixture was transformed into *E.coli* TOP-10 (Invitrogen) and selection was performed on Luria-agar plates containing 50 mg/L of carbenicillin and 20 mg/L of chloramphenicol. Plasmid was obtained and restriction enzyme analysis performed. Two plasmids, containing *loxP*-Cat-*loxP*-Trc with the *loxP*-Cat-*loxP* cassette in the same and in the opposite orientation relative to the *trc* promoter, were designated pTrCm41 and pTrCm42, respectively.

Construction of a *trc* promoter replacement template for *galP* (pR6KgalP).

A DNA cassette containing the *trc* promoter and *lac* operator with an upstream *loxP*-CAT-*loxP* cassette was PCR amplified from pTrCm42 using the primer pair SEQ ID NO:7 and SEQ ID NO:8. The primer pair incorporates 40 bp of homology to the *galP* upstream region to each end of the PCR product. PCR parameters were 95 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min, 30 cycles using *Taq* polymerase (Roche). The product was subcloned into Echo pUni/His5 R6K (Invitrogen) to generate the plasmid pR6KgalP.

Construction of a *trc* promoter replacement template for *glk* (pR6Kglk).

A DNA cassette containing the *trc* promoter and *lac* operator with an upstream *loxP*-CAT-*loxP* cassette was amplified from pTrCm42 by PCR using the primer pair SEQ ID NO:9 and SEQ ID NO:10. The primer pair SEQ ID NO:9 and SEQ ID NO:10 incorporates 39 (with a one base deletion) and 40 bp of homology, respectively, to the *glk* upstream region to each end of the PCR product. PCR parameters were 95 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min, 30 cycles using *Taq* polymerase (Roche).

The product was subcloned into Echo pUni/His5 R6K (Invitrogen) to generate the plasmid pR6Kglk.

Construction of an *E. coli* Δ*ptsHIcrr* strain (KLpts7).

A PTS minus derivative (Δ*ptsHIcrr*) of *E. coli* strain KLndh81 was obtained by P1 *vir* transduction using a derivative of *E. coli* strain NF9 as donor (Flores *et al., Nature Biotechnology* 14, 620-623 (1996)). The transduction replaces the operon comprising *ptsH, ptsI* and *crr* with a kanamycin antibiotic resistance marker (Levy *et al., Gene* 86, 27-33 (1990)) to give strain KLpts7. Plated on MacConkey (lactose$^-$) agar + 1% glucose, KLpts7 exhibits a white colony phenotype.

Replacement of the natural *galP* promoter with the synthetic *trc* promoter (KLgalP-trc).

A PCR amplification product comprising a *loxP*-Cat-*loxP*-Trc cassette and incorporating 40 bp of homology to the *galP* upstream region [for reference, see GenBank, Accession # U00096] to each end was generated using r*Tth* RNA polymerase (Perkin Elmer), pR6KgalP as the template and the primer pair SEQ ID NO:7 and SEQ ID NO:8. The PCR amplified integration cassette was transformed into electro-competent KLpts7 cells containing pKD46 for integration using the lambda Red system as described in Datsenko and Wanner, *supra*. Selection was performed on LB plates containing 10 mg/L chloramphenicol. Successful integration of this cassette replaces the region 38 to 181 bp upstream of the *galP* ATG start codon (for reference, see GenBank, Accession # U00096) with a *loxP*-Cat-*loxP*-Trc cassette (SEQ ID NO:11) to provide strain KLpts::galP-trc. Integration was confirmed by PCR analysis using the primer pair SEQ ID NO:7 and SEQ ID NO:8 (amplifying the integration site to give a 1.4 kb product) and the primer pair SEQ ID NO:12 and SEQ ID NO:13 (amplifying the integration site, including upstream and downstream regions, to give a 2.1 kb product). PCR parameters were 95 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min, 30 cycles using *Taq* polymerase or r*Tth* polymerase. KLpts::galP-trc, plated on MacConkey (lactose$^-$) agar + 1% glucose, exhibits a light red colony phenotype. The chloramphenicol marker was removed as described by Palmeros *et al., supra*. The removal was confirmed by PCR analysis using the primer pair SEQ ID NO:12 and SEQ ID NO:13 (to give a 1.1 kb product) and the resulting strain was designated KLgalP-trc.

Replacement of the natural *glk* promoter with the synthetic *trc* promoter (KLGG).

A PCR amplification product comprising a *loxP*-Cat-*loxP*-Trc cassette and incorporating approximately 40 bp of homology to the *glk* upstream region [for reference, see GenBank, Accession # U00096] to each end was generated using r*Tth* RNA polymerase (Perkin Elmer), pR6Kglk as the template, and the primer pair SEQ ID NO:9 and SEQ ID NO:10. The PCR amplified integration cassette was transformed into electro-competent KLgalP-trc cells containing pKD46 for integration using the lambda Red system as described above. Selection was performed on LB plates containing 10 mg/L chloramphenicol. Successful integration of this cassette replaces the region 40 to 137 bp upstream of the *glk* ATG start codon (for reference, see GenBank, Accession # U00096) with a *loxP*-Cat-*loxP*-Trc cassette (SEQ ID NO:11). Integration was confirmed by PCR analysis using the primer pair SEQ ID NO:14 and SEQ ID NO:15 (amplifying the integration site, including upstream and downstream regions, to give a 2.4 kb product). Plated onto MacConkey (lactose$^-$) agar + 1% glucose, colonies exhibit a deep red color, indicating an increase in the conversion of glucose to acid compared to the parent (KLgalP-trc). The chloramphenicol marker was removed as described above and subsequent PCR analysis (using the primer pair SEQ ID NO:14 and SEQ ID NO:15 to give a 1.3 kb product) gave the strain KLGG.

Deletion of the *arcA* gene, encoding a global regulator (KLGG ΔarcA).

A P1 lysate of the MG1655 ΔarcA::Cm strain was prepared and used to move the mutation to strain KLGG. A resulting chloramphenicol resistant clone, KLGG ΔarcA::Cm, was checked by genomic PCR to insure that the mutation was present. The chloramphenicol resistance marker was removed using the FLP recombinase (Datsenko and Wanner, *supra*) and this strain has been designated KLGG ΔarcA.

Deletion of the *edd* gene, encoding 6-phosphogluconate dehydrase gene (KLGG ΔarcA Δedd, also designated FMP).

An *edd* deletion [for reference, see GenBank, Accession # U00096] was obtained by replacing 1.7 kb of the coding region with a *loxP*-cat-*loxP* cassette from pLoxCat2. A replacement cassette was amplified with the primer pair SEQ ID NO:16 and SEQ ID NO:17. The primer SEQ ID NO:16 contains 80 bp of homology to the 5' end of *edd* and 18 bp of homology to the template pLoxCat2. The primer SEQ ID NO:17 contains 78 bp of

homology to the 3' end of *edd* and 19 bp of homology to pLoxCat2. The PCR product was gel-purified and electroporated into KLGG ΔarcA/pKD46 competent cells. Recombinant strains were selected on LB plates with 12.5 mg/L chloramphenicol. The deletion of the *edd* gene was confirmed by PCR using primer pair SEQ ID NO:18 and SEQ ID NO:19. The wild-type strain gives a 2.2 kb PCR product while the recombinant gives a characteristic 1.6 kb PCR product. This strain has been designated KLGG ΔarcA Δedd::cat. The chloramphenicol marker was removed using the Cre recombinase (Palmeros *et al., supra*) and this strain has been designated KLGG ΔarcA Δedd or, alternatively, FMP.

Selection and characterization of an FMP strain exhibiting an enhanced rate of glucose consumption.

Invariably, cells comprising Δ*ptsHIcrr*, the *trc* promoter replacement of the natural *galP* promoter, and the *trc* promoter replacement of the natural *glk* promoter (all three modifications as described in Example 4) initially exhibited slow growth. Also invariably, a subsequent selection (as described below) lead to a faster growing derivative. Glucokinase activity, assayed from cell-free extracts, was typically three-fold higher for the faster growing derivative as compared to the slower growing parent.

E. coli strain FMP, transformed with plasmid pSYCO103, was grown in a 14 L fermenter essentially as described in Example 2. Vials for storage at -80 °C (15 % glycerol stocks) were made over the course of the fermentation. A LB plate was streaked from the aliquot taken at 33 hrs (OD_{550} was 30.7 AU) and single colonies were recovered and designated "selected FMP/pSYCO103". Single colonies were similarly obtained from strain FMP containing no plasmid and designated "selected FMP".

The *galP* and *glk* genes, including the introduced *trc* promoter region, were PCR amplified from two colonies of "selected FMP/pSYCO103" and one colony of "selected FMP" using the primer pair SEQ ID NO:12/SEQ ID NO:13 and the primer pair SEQ ID NO:14/SEQ ID NO:15, respectively. Sequence analysis on these three samples was performed using the same primers. In each case, the *galP* gene and promoter region remained unchanged from the parent strain while the *glk* gene and promoter region contained an identical, single base pair change when compared the parent strain. The two isolates of "selected FMP/pSYCO103" and one isolate of "selected FMP" contained the sequence identified as SEQ ID NO:20 in the – 35 to –10 region of *trc*

promoter controlling *glk* expression compared to the corresponding parent sequence SEQ ID NO:21. The strain originating from "selected FMP" from which *galP* and *glk* sequence was obtained was designated strain FMP'::Km.

EXAMPLE 5

COMPARATIVE EXAMPLES OF GLUCOSE TO 1,3-PROPANEDIOL FERMENTATIONS USING STRAINS FMP/pSYCO103 AND FMP'::Km/pSYCO103

Strains FMP and FMP'::Km were transformed with the plasmid pSYCO103. Production of 1,3-propanediol was determined in 14 L fermenters as described in General Methods with the following differences in the control parameters or the fermenter. A thawed frozen vial of FMP/pSYCO103 was transferred to 500 mL 2YT with 50 mg/L spectinomycin to prepare the pre-culture. The dO set-point was 15%. Vitamin B_{12} was added to the fermenter in 16 mg boluses at 30, 43, and 51 h.

A thawed frozen vial of FMP'::Km/pSYCO103 was transferred to 500 mL SBG1% with 50 mg/L spectinomycin to prepare the pre-culture. Vitamin B_{12} was added to the fermenter in 16 mg boluses at 21, 40 and 40.5 h.

Figure 1 shows the production of 1,3-propanediol by FMP'::Km/pSYCO103 is faster than by FMP/pSYCO103.

EXAMPLE 6

ENGINEERING ALTERED EXPRESSION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (*gapA*) IN *E. COLI* STRAINS FOR PRODUCTION OF 1, 3 PROPANEDIOL FROM GLUCOSE

Decreasing GapA expression by altering the start condon.

The level of glyceraldehyde-3-phosphate dehydrogenase, GapA, was decreased by replacing the ATG start codon of the *gapA* gene with a GTG or TTG start codon.

The *E. coli gapA* gene plus upstream and downstream flanking sequence was amplified by PCR from *E. coli* strain MG1655 using primer pair SEQ ID NO:22 and SEQ ID NO:23. The PCR product was cloned directly from the PCR reaction into pCR-BluntII-TOPO (Invitrogen) to form pDT50. The plasmids pDT50 and pLitmus 28 (New England Biolabs, Inc.) were digested with *Sph*I and *Bam*H I and the *gapA* gene fragment and

vector, respectively, were gel-purified and ligated. The resulting plasmid, pDT51, was transformed into *E. coli* TOP10 (Invitrogen).

The *gapA* mutant plasmids were constructed using the Stratagene QuickChange 1-Day Site-Directed Mutagenesis Method (Stratagene, La Jolla, CA). The template plasmid, pDT51, was mixed with either the primer pair SEQ ID NO:24 and SEQ ID NO:25 to create the GTG mutation or the primer pair SEQ ID NO:26 and SEQ ID NO:27 to create the TTG mutation. After the PCR amplification, the reactions were digested with *Dpn*I to remove the template plasmid and leave only the amplified plasmids. The plasmids were then transformed into *E. coli* TOP10 (Invitrogen).

The *gapA*-GTG and *gapA*-TTG constructs were PCR amplified using primer pair SEQ ID NO:22 and SEQ ID NO:23. The PCR products were electroporated into the *gapA* knockout strain, KLP23A112. *E. coli* strain KLP23A112 was constructed by transducing KLP23 with a P1 phage lysate obtained from *E. coli* DS112 (*E. coli* Genetic Stock Center), a *gapA* deletion strain containing a CmR marker. Recombinants were selected for growth on LB plates without added glucose and sensitivity to chloramphenicol. Sequencing confirmed the successful integration of the GTG and TTG mutations. The mutated strains were named KLPAGTG and KLPATTG, respectively. Measurement of the GapA activities in the KLPAGTG and KLPATTG strains showed that the GapA levels were 4% and <1 % of the control strain KLP23, respectively.

Strains KLP23, KLPAGTG, and KLPATTG were transformed with the plasmid pSYCO101 and tested for 1,3-propanediol production in TM2 medium containing 40 g/L glucose, 50 mg/L spectinomycin, and 1 mg/L vitamin B_{12}. The results are shown in Table 4.

TABLE 4

Representative shake flasks summary of the conversion of glucose to 1,3-propanediol using *E. coli* strains KLP 23/pSYCO101, KLP AGTG/pSYCO101, and KLPATTG/pSYCO101

Strain	Glycerol (g/L)	1,3-Propanediol (g/L)	Molar Yield (mol/mol)
KLP23 pSYCO101	5.8	10.7	0.87
KLPAGTG pSYCO101	0.7	1.3	0.11
KLPATTG pSYCO101	0.2	0.4	0.04

Altering GapA expression by replacing the promoter.

Replacement of the natural *gapA* promoter with the synthetic short 1.5 GI promoter (SEQ ID NO:28) was made by replacing 225 bp of upstream *gapA* sequence [for reference, see GenBank, Accession # U00096) with FRT-CmR-FRT and an engineered promoter. The replacement cassette was amplified by PCR with the primer pair SEQ ID NO:29 and SEQ ID NO:30 using pKD3 as a template. The primer SEQ ID NO:29 contains 39 bp of homology to *gapA* including the ATG start, contains the short 1.5 GI promoter and contains 20 bp of homology to template pKD3. Primer SEQ ID NO:30 contains 59 bp of homology to upstream *gapA* sequence and 21 bp of homology to pKD3. The PCR products were gel-purified and electroporated into MG1655/pKD46 competent cells to give MG1655 1.5gapA::Cm. Recombinant strains were selected on LB plates with 12.5 mg/L chloramphenicol. Successful integration of the cassette replaces the region 34-258 bp upstream of the *gapA* ATG start codon with a FRT-CmR-FRT-short 1.5 GI promoter cassette. A P1 phage lysate was prepared and used to move the mutation to FMP'::Km. This strain was designated FMP'::Km 1.5gapA::Cm.

The short 1.5 GI *gapA* promoter in MG1655 1.5gapA::Cm was replaced with the short 1.20 GI promoter (SEQ ID NO:31) or the short 1.6 GI promoter (SEQ ID NO:32). To create the1.20 gapA strain, a replacement cassette was PCR amplified with primer pair SEQ ID NO:33 and SEQ ID NO:34 using genomic DNA from MG1655 1.5gapA::Cm as template. Primer SEQ ID NO:33 contains 24 bp of homology to the *gapA* upstream region. Primer SEQ ID NO:34 contains homology to the *gapA* upstream region in MG1655 1.5gapA::Cm and contains the short 1.20 GI promoter. To create the 1.6gapA strain, a replacement cassette was PCR amplified with primer pair SEQ ID NO:33 and SEQ ID NO:35 using genomic DNA from MG1655 1.5gapA::Cm as template. The primer SEQ ID NO:35 contains homology to the *gapA* upstream region in MG1655 1.5gapA::Cm and contains the short 1.6 GI promoter. The short 1.20 GI promoter replacement cassette and the short 1.6 GI promoter replacement cassette were used to replace the natural *gapA* promoter of MG1655 as described above to give strains MG1655 1.20gapA::Cm and MG1655 1.6gapA::Cm, respectively. MG1655 1.20gapA::Cm and

MG1655 1.6gapA::Cm were used to replace the natural *gapA* promoter of strain FMP'::Km (using P1 tranduction as described above) to give strains FMP'::Km 1.20gapA::Cm and FMP'::Km 1.6gapA::Cm, respectively.

Glyceraldehyde-3-phosphate dehydrogenase activities were determined using cell-free extracts prepared from the strains FMP'::Km 1.20gapA::Cm, FMP'::Km 1.5gapA::Cm, FMP'::Km 1.6gapA::Cm and FMP'::Km as control. The values obtained, compared to that of control, were 10%, 25 % and 140 % for strains FMP'::Km 1.20gapA::Cm, FMP'::Km 1.5gapA::Cm, FMP'::Km 1.6gapA::Cm, respectively.

The strains containing the GI promoter replacements were transformed with the plasmid pSYCO106 and compared to the parent strain for 1,3-propanediol production in TM2 medium containing 20 g/L glucose, 50 mg/L spectinomycin, and 1 mg/L vitamin B_{12}. The results are shown in Table 5.

TABLE 5

Representative shake flasks summary of FMP'::Km GI promoter gapA strains transformed with the plasmid pSYCO106

Strain	Glycerol (g/L)	1,3-Propanediol (g/L)	Molar yield (mol/mol)
FMP'::Km/pSYCO106	1.8	6.8	1.07
FMP'::Km 1.6gapA/pSYCO106	4.1	3.3	0.92
FMP'::Km 1.5gapA/pSYCO106	6.6	3.6	1.21
FMP'::Km 1.20 gapA/pSYCO106	7.6	2.8	1.26

EXAMPLE 7

REMOVING THE MARKERS FROM FMP'::Km 1.5gapA::Cm.

The chloramphenicol marker was removed from strain FMP'::Km 1.5gapA::Cm (as described in the General Methods section) to give strains FMP'::Km 1.5gapA.

The kanamycin marker introduced into FMP'::Km 1.5gapA as a consequence of making KLndh81 PTS minus (ΔptsHIcrr) was replaced with a removable FRT-Cm-FRT cassette by P1 transduction from MG1655 ΔptsHIcrr::Cm. A *ptsHIcrr* deletion in MG1655 was made with a replacement cassette amplified with primer pair SEQ ID NO:54 and SEQ ID NO:55 using pKD3 as template. The primer SEQ ID NO:54 contains 78 bp of homology to the remaining region of *ptsH* left in the chromosome

of strain FMP'::Km 1.5gapA and 20 bp of homology to pKD3. The primer SEQ ID NO:55 contains 77 bp of homology to the remaining region of *crr* in strain FMP'::Km 1.5gapA and 20 bp of homology to pKD3. The PCR products were gel-purified and electroporated into MG1655/pKD46 competent cells. Recombinant strains were selected on LB plates with chloramphenicol 12.5 mg/L. PCR analysis confirmed the integration of the cassette. Plated on MacConkey (lactose$^-$) agar + 1% glucose, MG1655 ΔptsHIcrr::Cm exhibits a white colony phenotype. A P1 phage lysate was prepared and the Cm marker transduced into FMP'::Km 1.5gapA. Recombinant strains were selected on chloramphenicol and PCR analysis confirmed successful integration of the cassette. The chloramphenicol marker was removed using the Flp recombinase and sequencing (using primer SEQ ID NO:56) confirmed the removal of the chloramphenicol marker. The resulting strain was designated FMP' 1.5 gapA.

EXAMPLE 8

DEMONSTRATING HIGH YIELD OF 1,3-PROPANEDIOL FROM GLUCOSE USING *E. COLI* STRAIN FMP' 1.5gapA/pSYCO106

Strain FMP' 1.5gapA was transformed with the plasmid pSYCO106. Production of 1,3-propanediol and glycerol was determined in 14 L fermenters as described in General Methods with the following differences in the control parameters for the fermenter. A thawed frozen vial of FMP' 1.5gap/pSYCO106 was transferred to 500 mL SBG1% with 50 mg/L spectinomycin to prepare the pre-culture. Vitamin B_{12} was added to the fermenter in 16 mg boluses prior to inoculation and at 28 hrs. Final 1,3-propanediol concentration was 129 g/L and the mass yield was 40.2%.

EXAMPLE 9

ENGINEERING A METHYLGLYOXAL SYNTHASE (*mgsA*) MUTANT IN *E. COLI*

The *mgsA* deletions [for reference, see GenBank, Accession # U00096] were made by replacing 0.4 kb of the coding region with the FRT-Kan-FRT cassette of pKD4. A replacement cassette was PCR amplified with the primer pair SEQ ID NO:36 and SEQ ID NO:37 using pKD4 as the template. The primer SEQ ID NO:36 contains 40 bp of homology to the 5' end of *mgsA* and 20 bp of homology to the template DNA, pKD4. The primer SEQ ID NO:37 contains 40 bp of homology to the 3' end of *mgsA* and 20 bp of homology to pKD4. The PCR product was gel-purified and electroporated into MG1655/pKD46 competent cells.

Recombinant strains were selected on LB plates with 12.5 mg/L of kanamycin. The deletion of the *mgsA* gene was confirmed by PCR, using the primer pair SEQ ID NO:38 and SEQ ID NO:39. The wild-type strain gives a 1.3 kb PCR product while the recombinant strain gives a characteristic 2.4 kb PCR product. This strain has been designated MG1655 Δ*mgsA::kan*. Once the *mgsA* mutant was obtained in MG1655, a P1 phage lysate was prepared and used to move the mutation into FMP' 1.5gapA (Example 8). The kanamycin resistance marker was removed using the FLP recombinase (Datsenko and Wanner, *supra*) and this strain has been designated FMP' 1.5gapA ΔmgsA.

FMP' 1.5gapA ΔmgsA and its parent were transformed with the plasmid pSYCO106 to give FMP' 1.5gapA ΔmgsA/pSYCO106 and FMP' 1.5gapA/pSYCO106, respectively.

The strains were tested for 1,3-propanediol production in TM2 medium containing 20 g/L glucose, 50 mg/L spectinomycin, and 1 mg/L vitamin B_{12}. The results are shown in Table 6.

TABLE 6

Representative shake flasks summary of FMP' 1.5gapA/pSYCO106 and FMP' 1.5gapA ΔmgsA/pSYCO106 strains

Strain	Glycerol (g/L)	1,3-Propanediol (g/L)	Molar yield (mol/mol)
FMP'1.5gapA/pSYCO106	6.6	3.6	1.21
FMP'1.5gapA ΔmgsA/pSYCO106	8.3	2.4	1.26

EXAMPLE 10

FERMENTATION OF GLUCOSE TO 1,3-PROPANEDIOL USING *E. COLI* STRAIN FMP' 1.5gapA ΔmgsA /pSYCO106

Strain FMP' 1.5gapA ΔmgsA was transformed with the plasmid pSYCO106. Production of 1,3-propanediol (and glycerol) was determined in 14 L fermenters as described in General Methods with the following differences in control parameters for fermenters. A single colony from a fresh plate (LA with 50 mg/L spectinomycin) of FMP' 1.5gapA ΔmgsA /pSYCO106 was transferred to 30 mL LB with 100 mg/L spectinomycin in a 250 mL flask to prepare the pre-culture. After incubation at 34 °C and 300 rpm to an OD_{550} of 1 AU, 10.8 mL of the culture was transferred to the

fermenter. The fermenter was run with glucose limitation during much of the run. Vitamin B_{12} was added to the fermenter in 16 mg boluses prior to inoculation, at 28 hrs and at 38 hrs. Final 1,3-propanediol concentration was 130 g/L and the mass yield was 47.5%. A run with glucose maintained in excess (0-20 g/L) gave 141 g/L 1,3-propanediol and a mass yield of 43.6%.

EXAMPLE 11

CONSTRUCTION OF AN *E. COLI* STRAIN WITH AN ENGINEERED PROMOTER FOR PHOSPHOENOLPYRUVATE CARBOXYLASE (*ppc*) BY LINEAR DNA TRANSFORMATION

Replacement of the natural *ppc* promoter with the synthetic short 1.6 GI promoter was made by replacing 59 bp of the upstream *ppc* sequence cassette containing FRT-CmR-FRT and an engineered promoter. The PCR product was amplified with the primer pair SEQ ID NO:40 and SEQ ID NO:41 using pKD3 as the template. Primer SEQ ID NO:40 contains 80 bp of homology to upstream *ppc* sequence and 20 bp of homology to template pKD3. Primer SEQ ID NO:41 contains 39 bp of homology to upstream *ppc* sequence, contains the short 1.6 GI promoter sequence and contains 20 bp of homology to pKD3. The PCR products were gel-purified and electroporated into MG1655/pKD46 competent cells. Recombinant strains were selected on LB plates with 12.5 mg/L chloramphenicol to give MG1655 1.6ppc::Cm. Successful integration of the cassette replaces the region 90 to 148 bp upstream of the *ppc* ATG start [for reference, see GenBank, Accession # U00096] with a FRT-CmR-FRT-short 1.6 GI promoter cassette. Integration into the upstream *ppc* region was confirmed by primer pair SEQ ID NO:40 and SEQ ID NO:41. The wild-type strain gives a 0.2 kb PCR product while the recombinant strain gives a characteristic 1.2 kb PCR product. This PCR product was sequenced using primer SEQ ID NO:42, which indicated that the promoter replacement effectively occurred. A P1 phage lysate was prepared and used to move the mutation to strain FMP'1.5gap ΔmgsA. This strain was designated FMP'1.5gap Δmgs 1.6ppc::Cm. The chloramphenicol-resistance marker was removed using the FLP recombinase (Datsenko and Wanner, *supra*), and the resulting strain was electrotransformed with plasmid pSYCO106 to give FMP'1.5gap Δmgs 1.6ppc/pSYCO106.

Shake flask cultures were used to assess the conversion of glucose to 1,3-propanediol in *E. coli* strains FMP'1.5gap Δmgs/pSYCO106 and

FMP'1.5gap Δmgs 1.6ppc/pSYCO106. The strains, grown in LB medium containing 50 mg/L spectinomycin for 10 hrs, were used to inoculated (200 μL) into 250 mL-baffled Erlenmeyer flasks containing 10 mL TM2 medium, 20 g/L glucose, 50 mg/L spectinomycin, and 2 mg/L vitamin B_{12}. The flasks were incubated at 300 rpm and 34 °C. Representative results are given in Table 7. Both an increase in the molar yield and a decrease of acetate production were observed with the addition of the 1.6ppc mutation to the parent strain.

TABLE 7

Shake Flasks for Conversion of Glucose to 1,3-Propanediol (1,3-PD)

Strain	Glycerol produced (g/L)	1,3-Propanediol produced (g/L)	Acetate produced (g/L)	Molar Yield
FMP'1.5gap ΔmgsA/pSYCO106	8.24	2.19	1.78	1.25
FMP'1.5gap Δmgs 1.6ppc/pSYCO106	7.5	3.34	0.34	1.32

Phosphoenolpyruvate carboxylase (Ppc) activity was measured from cell-free extracts obtained from the shake flasks described immediately above. Aliquots of cells were harvested in mid-log phase, broken by two passages through a French press cell, centrifuged for 15 min at 14,000 rpm, and ultracentrifuged 1 hr at 50,000 rpm. The supernatant was removed and used as a source of proteins. Specific activities of PPC are reported in Table 8 below. The replacement of the natural ppc promoter with the short 1.6 GI promoter increased the Ppc enzyme activity three-fold.

TABLE 8

PPC Enzyme Specific Activity

Strain	Phosphoenolpyruvate carboxylase specific activity (U/mg protein)
FMP'1.5gap ΔmgsA, pSYCO106	0.28
FMP'1.5gap Δmgs 1.6ppc, pSYCO106	0.86

EXAMPLE 11A

FERMENTATION OF GLUCOSE TO 1,3-PROPANEDIOL USING *E. COLI* STRAIN FMP' 1.5gapA ΔmgsA /pSYCO106

Production of 1,3-propanediol by FMP' 1.5gapA ΔmgsA 1.6ppc/pSYCO106 was determined in 14 L fermenters as described in General Methods with the following differences in control parameters for fermenters. A single colony from a fresh plate (LA with 50 mg/L spectinomycin) of FMP' 1.5gapA ΔmgsA 1.6ppc/pSYCO106 was transferred to 30 mL LB with 100 mg/L spectinomycin in a 250 mL flask to prepare the pre-culture. After incubation at 34 °C and 300 rpm to an OD550 of 1 AU, 10.8 mL of the culture was transferred to the fermenter. Vitamin B_{12} was added to the fermenter in 16 mg boluses prior to inoculation, at 28 hrs, and at 38 hrs. Final 1,3-propanediol concentration was 135.3 g/L and the mass yield was 46.1%.

EXAMPLE 12

CONSTRUCTION OF *E. COLI* STRAIN WITH AN ENGINEERED PROMOTER FOR *yciK/btuR* BY LINEAR DNA TRANSFORMATION

The genes *yciK* and *btuR* are present within a single operon in *E. coli*. Replacement of the natural *yciK-btuR* promoter with the synthetic short 1.6 GI promoter was made by inserting a 1.3 kb cassette, upstream of *yciK-btuR*. The replacement cassette, containing FRT-CmR-FRT and an engineered promoter, was amplified by PCR with the primer pair SEQ ID NO:43 and SEQ ID NO:44 using pKD13 as the template. Primer SEQ ID NO:43 contains 70 bp of homology to upstream *yciK-btuR* sequence and 20 bp of homology to template pKD13. Primer SEQ ID NO:44 contains 30 bp of homology to upstream *yciK-btuR* sequence, contains the short 1.6 GI promoter sequence and contains 20 bp of homology to pKD13. The PCR products were gel-purified and electroporated into MG1655/pKD46 competent cells. Recombinant strains were selected on LB plates with 25.0 mg/L kanamycin to give MG1655 1.6yciK-btuR::Km. Successful integration of the cassette results in an insertion between bp 27 and bp 28 upstream of the *yciK* ATG start codon [for reference, see GenBank, Accession # U00096] with a FRT-CmR-FRT-short 1.6 GI promoter cassette. Integration into the upstream *yciK/btuR* region was confirmed by primer pair SEQ ID NO:45 and SEQ ID NO:46. The wild-type strain gives a 1.4 kb PCR product while the recombinant strain gives

a characteristic 2.8 kb PCR product. A P1 phage lysate was prepared and used to move the mutation to a derivative of strain FMP'1.5gap Δmgs 1.6ppc named Triple. After removing the antibiotic, strain Triple 1.6btuR was obtained.

EXAMPLE 12A

FERMENTATION OF GLUCOSE TO 1,3-PROPANEDIOL USING *E. COLI* STRAIN Triple 1.6btuR /pSYCO109

Strain Triple 1.6btuR was transformed with pSYCO109. Production of 1,3-propanediol by Triple 1.6btuR /pSYCO109 was determined in 14 L fermenters as described in General Methods with the following differences in control parameters for fermentation. A single colony from a fresh plate (LA with 50 mg/L spectinomycin) of Triple 1.6btuR /pSYCO109 was transferred to 30 mL LB with 100 mg/L spectinomycin in a 250 mL flask to prepare the pre-culture. After incubation at 34 °C and 300 rpm to an OD550 of 1 AU, 10.8 mL of the culture was transferred to the fermenter. Vitamin B_{12} was added to the fermenter in 8 mg boluses prior to inoculation, at 28 hrs, and at 38 hrs. Final 1,3-propanediol concentration was 123 g/L and the mass yield was 45.7%.

EXAMPLE 13

CONSTRUCTION OF *E. COLI* STRAIN WITH AN ENGINEERED PROMOTER FOR *yqhD* BY LINEAR DNA TRANSFORMATION

Replacement of the natural *yqhD* (alcohol dehydrogenase) promoter with the synthetic short 1.6 GI promoter was made by replacing 967 bp of upstream *yqhD* sequence including the *yqhC* gene with a cassette containing FRT-CmR-FRT and an engineered promoter. The PCR product was amplified with the primer pair SEQ ID NO:47 and SEQ ID NO:48 using pKD3 as the template. Primer SEQ ID NO:47 contains 78 bp of homology to upstream *yqhD* sequence and 20 bp of homology to template pKD3. Primer SEQ ID NO:48 incorporates 41 bp of homology (with a 1 bp deletion) to upstream *yqhD* sequence, 40 bp of homology to the short 1.6 GI promoter sequence, and 19 bp of homology to pKD3. The PCR products were gel-purified and electroporated into MG1655/pKD46 competent cells. Recombinant strains were selected on LB plates with 12.5 mg/L chloramphenicol to give MG1655 1.6yqhD::Cm. Successful integration of the cassette replaces the region 50-1016 bp upstream of the *yqhD* ATG start [for reference, see GenBank, Accession # U00096] with a FRT-CmR-FRT-short 1.6 GI promoter cassette.

Integration into the upstream *yqhD* region was confirmed by sequencing with primer SEQ ID NO:49 and indicated that the promoter replacement effectively occurred. A P1 phage lysate was prepared and used to move the mutation into strain Triple 1.6btuR. After removing the antibiotic (as described above), strain Triple 1.6btuR 1.6yqhD was obtained.

EXAMPLE 13A

FERMENTATION OF GLUCOSE TO 1,3-PROPANEDIOL USING *E. COLI* STRAIN Triple 1.6btuR 1.6yqhD/pSYCO109

Strain Triple 1.6btuR 1.6yqhD was transformed with pSYCO109. Production of 1,3-propanediol by Triple 1.6btuR 1.6yqhD/pSYCO109 was determined in 14 L fermenters as described in General Methods with the following differences in control parameters for fermentation. A single colony from a fresh plate (LA with 50 mg/L spectinomycin) of Triple 1.6btuR 1.6yqhD/pSYCO109 was transferred to 30 mL LB with 100 mg/L spectinomycin in a 250 mL flask to prepare the pre-culture. After incubation at 34 °C and 300 rpm to an OD550 of 1 AU, 10.8 mL of the culture was transferred to the fermenter. Vitamin B_{12} was added to the fermenter in a single 16 mg bolus at 20.6 hrs elapsed fermentation time. Final 1,3-propanediol concentration was 113.3 g/L and the mass yield was 48.8%.

EXAMPLE 14

CONSTRUCTION OF AN *E. COLI* STRAIN WITH A DELETION MUTATION IN ACETATE KINASE (*ack*) AND PHOSPHOTRANSACETYLASE (*pta*) BY LINEAR DNA TRANSFORMATION

The *pta-ackA* deletion [for reference, see GenBank, Accession # U00096] was made by replacing 3.3 kb of the coding region with the FRT-CmR-FRT cassette of pKD3. The replacement cassette was amplified with the primer pair SEQ ID NO:50 and SEQ ID NO:51 using pKD3 as the template. The primer SEQ ID NO:50 contains 80 bp of homology to the 5' end of *pta* and 20 bp of homology to the template DNA, pKD3. The primer SEQ ID NO:51 contains 80 bp of homology to the 3' end of *ackA* and 20 bp of homology to pKD3. The PCR products were gel-purified and electroporated into MG1655/pKD46 competent cells. Recombinant strains were selected on LB plates with 12.5 mg/L of chloramphenicol to give strain MG1655 ΔackA-pta::Cm. The deletion of the *pta-ackA* genes was confirmed by PCR, using the primer pair SEQ ID NO:52 and SEQ ID

NO:53. The wild-type strain gives a 3.8 kb PCR product while the recombinant strain gives a characteristic 1.6 kb PCR product. A P1 phage lysate was prepared and used to pass the mutation to strain Triple 1.6btuR 1.6yqhD to form strain Triple 1.6btuR 1.6yqhD ΔackA-pta::Cm. The chloramphenicol resistance marker was removed using the FLP recombinase (Datsenko and Wanner, *supra*) to give Triple 1.6btuR 1.6yqhD ΔackA-pta (renamed TripleTriple (TT)). The Triple 1.6btuR 1.6yqhD and TT strains were electrotransformed with plasmid pSYCO109.

EXAMPLE 15

MEASUREMENT OF PHOSPHOTRANSACETYLASE (Pta) ENZYME ACTIVITY IN STRAIN TRIPLE TRIPLE/pSYCO109 COMPARED TO STRAIN TRIPLE 1.6btuR 1.6yqhD /pSYCO109

Fermentations with TT/pSYCO109 and Triple 1.6btuR 1.6yqhD/pSYCO109 were carried out in 14L fermenters as described in the General Methods with the following differences in control parameters for fermentation. A typical fermentation with Triple 1.6btuR 1.6yqhD/pSYCO106 was described in Example 13A.

A pre-culture of TT/pSYCO109 was grown in 30 mL LB with 100 mg/L spectinomycin in a 250 mL flask to an OD550 of approximately 1 AU. A seed fermenter prepared as described was inoculated with 10.8 mL of that culture. After 30.5 hrs of fermentation time, 1.2 L of the culture was transferred to a production fermenter. This fermenter received a single 16 mg bolus of vitamin B_{12} 1 hr after inoculation. The final concentration of 1,3-propanediol in a typical fermentation was 114 g/L and the yield was 48%.

Samples from a typical Triple 1.6btuR 1.6yghD/pSYCO109 fermentation were analyzed for Pta enzyme activity. A specific activity of 0.4 U/mg protein was obtained. Samples assayed from a typical TT/pSYCO109 fermentation showed no detectable Pta enzyme activity.

EXAMPLE 16

IMPROVED STABILITY OF MOLAR YIELDS IN STRAIN TT/pSYCO109 COMPARED TO TRIPLE 1.6btuR 1.6yqhD/pSYCO109

Duplicate shake flasks cultures were grown with strains Triple btuR 1.6 yqhD, pSYCO109, and TT pSYCO109. After incubating one colony for 10 hr in LB + 50 mg/L spectinomycin, 100 mL of culture were transferred in 30 mL TM2 medium with 2% glucose and with or without 50 ppm spectinomycin (day 1). In order to study the stability of the yield, a

100 mL volume of the day 1 cultures was transferred after 24 hr to a fresh volume of 30 mL TM2 media containing 2% glucose with or without 50 ppm spectinomycin. This was repeated 4 times. The molar yield was calculated as in Example 2 at the end of each 24 hr period and results are given below in Table 9. The *ackA-pta* deletion stabilizes the molar yield, therefore improving 1,3-propanediol production.

TABLE 9

		Molar Yield				
Strain	With (+) or Without (-) Spetinomycin	Molar Yield Day 1	Molar Yield Day 2	Molar Yield Day 3	Molar Yield Day 4	Molar Yield Day 5
Triple btuR, 1.6yqhD, pSYCO109	+	1.24	1.28	1.06	0.84	0.79
TT, pSYCO109	+	1.15	1.22	1.24	1.24	1.07
Triple btuR 1.6yqhD, pSYCO109	-	1.23	1.04	0.95	0.61	0.25
TT, pSYCO109	-	1.23	1.22	1.23	1.13	1.11

EXAMPLE 17

STRUCTION OF *E. COLI* STRAIN WITH DELETION MUTATIONS IN ALDEHYDE DEHYDROGENASES BY LINEAR DNA TRANSFORMATION

An *aldA* deletion [for reference, see GenBank, Accession # U00096] was made by replacing 1.3 kb of the coding region with the FRT-CmR-FRT cassette of pKD3. The cassette was amplified with the primer pair SEQ ID NO:57 and SEQ ID NO:58 using pKD3 as the template. The primer SEQ ID NO:57 contains 80 bp of homology to the 5' end of *aldA* and 20 bp of homology to the template DNA, pKD3. The primer SEQ ID NO:58 contains 80 bp of homology to the 3' end of *aldA* and 20 bp of homology to pKD3. The PCR products were gel-purified and electroporated into MG1655/pKD46 competent cells. Recombinant strains were selected on LB plates with 12.5 mg/L of chloroamphenicol. The deletion of the *aldA* gene was confirmed by PCR, using the primer pair SEQ ID NO:59 and SEQ ID NO:60. The wild-type strain gives a 2.0 kb PCR product while the recombinant strain gives a characteristic 1.8 kb PCR product. A P1 lysate of that strain was prepared and used to move

the mutation to the strain TT to form the TT ΔaldA::Cm strain. The chloramphenicol resistance marker was removed using the FLP recombinase (Datsenko and Wanner, *supra*) to create TT aldA.

An *aldB* deletion [for reference, see GenBank, Accession # U00096] was made by replacing 1.5 kb of the coding region with the FRT-CmR-FRT cassette of the pKD3. A replacement cassette was amplified with the primer pair SEQ ID NO:61 and SEQ ID NO:62 using pKD3 as the template. The primer SEQ ID NO:61 contains 80 bp of homology to the 5'-end of *aldB* and 20 bp of homology to pKD3. Primer SEQ ID NO:62 contains 80 bp of homology to the 3' end of *aldB* and 20 bp homology to pKD3. The PCR products were gel-purified and electroporated into MG1655/pKD46 competent cells. Recombinant strains were selected on LB plates with 12.5 mg/L of chloroamphenicol. The deletion of the *aldB* gene was confirmed by PCR, using the primer pair SEQ ID NO:63 and SEQ ID NO:64. The wild-type strain gives a 1.5 kb PCR product while the recombinant strain gives a characteristic 1.1 kb PCR product. A P1 lysate was prepared and used to move the mutation to the TT strain to form the TT ΔaldB::Cm strain. A chloramphenicol-resistant clone was checked by genomic PCR with the primer pair SEQ ID NO:63 and SEQ ID NO:64 to insure that the mutation was present.

EXAMPLE 17A

PRODUCTION OF GLYCEROL WITH STRAIN FMP' 1.5gap/pSYCO106

Strain FMP' 1.5gapA was transformed with the plasmid pSYCO106. Production of glycerol was determined in 14 L fermenters as described in General Methods with the following differences in control parameters for fermentation. A thawed frozen vial of FMP' 1.5gap/pSYCO106 was transferred to 500 mL SBG1% with 50 mg/L spectinomycin to prepare the pre-culture. No vitamin B_{12} was added to the fermenter. A typical fermentation resulted in the production of 202 g/L glycerol with a molar yield of 115%.

EXAMPLE 17B

PRODUCTION OF GLYCEROL WITH STRAIN TT/pSYCO109

Production of glycerol was determined in 14 L fermenters as described in General Methods with the following differences in control parameters for fermentation. A single colony of TT/pSYCO109 on a LB plate with 50 mg/L spectinomycin was transferred to 30 mL LB with 100 mg/L spectinomycin in a 250 mL flask. When an OD550 of approximately

1 Au was reached, 10.8 mL of the culture was used to inoculate a fermenter prepared as described. No vitamin B_{12} was added to the fermenter. A typical fermentation resulted in the production of 302 g/L glycerol with a molar yield of 137%.

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